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# AN ELECTROPHORETIC SURVEY OF VARIOUS ANIMAL PLASMAS

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*(Received for publication, June 7, 1945)*

Previous electrophoretic studies have been reported on the plasma and serum proteins of man and of various animals, often in connection with a pathology involving the blood proteins of the animal in question. However, among the animals studied, there seldom exists a strict basis of comparison, since different investigators have carried out their experiments in different buffers of varying pH and ionic strength. Moreover, various optical devices have been used to resolve the protein concentration gradients.

It was the purpose of this investigation to determine the electrophoretic constituents of plasma, under identical conditions in so far as possible, of a variety of easily accessible animal species in order to ascertain differences in composition. Extensive studies of any given animal or determinations made under varying conditions were not attempted. In the species of animals examined, a complexity and variation of proteins that are both interesting and provocative of thought have been revealed.

## EXPERIMENTAL

Whenever possible, food was withheld overnight from animals prior to bleeding in order to minimize plasma turbidities due largely to lipemia. Sufficient blood was removed to permit an electrophoretic analysis for an individual animal. 5 to 8 volumes of blood, usually obtained by cardiac puncture, were immediately mixed with 1 volume of isotonic sodium oxalate (0.1 M). After centrifugation, the supernatant plasma was removed with a pipette and diluted to a protein concentration of 2.5 to 3.5 per cent, the higher protein range being used for samples low in globulins to provide sufficient areas under the curves to permit a better evaluation of their boundaries. The samples were placed in bags of Visking tubing and dialyzed with agitation for 20 to 60 hours at 0° against two changes of a buffer, 0.05 M in diethylbarbituric acid, 0.00765 M in sodium citrate, and adjusted to pH 8.6 with sodium hydroxide.<sup>1</sup> After dialysis the samples were centrifuged briefly to remove any cellular debris that may have been introduced into the dialysis bag. The conductivity of the final dialysate was measured by means of a Wheatstone bridge assembly, with the conductance cell mounted in the electrophoresis bath.

<sup>1</sup> The veronal buffer of Longworth (1), modified by the addition of sodium citrate.

TABLE I  
Per Cent Composition of Plasma of Various Species

Species	No. of plasmas examined	Fig. No.	$\gamma$	$\phi$	$\beta$	$\alpha_3$	$\alpha_2$	$\alpha_1$	Albumin	$f$	Albu- min- glo- bulin ratio	Component with which turbidity moved
Man.....	3	1	9.1 $\pm$ 0.2*	4.8 $\pm$ 0.2	11.0 $\pm$ 1.1	4.7	8.8 $\pm$ 0.4	6.7 $\pm$ 0.5	59.6 $\pm$ 0.7	0.5 $\pm$ 0.1	1.5	$\beta + \alpha_2$
Monkey..	3	2	9.0 $\pm$ 0.4	8.4 $\pm$ 0.0	16.1 $\pm$ 0.9	$\pm$ 0.5	5.2 $\pm$ 0.5	5.9 $\pm$ 1.0	50.0 $\pm$ 0.5		1.0	" + $\alpha_3$ , mostly $\alpha_3$
Horse....	4	3	11.2 $\pm$ 0.3	15.8 $\pm$ 0.4	21.9 $\pm$ 1.0		12.3 $\pm$ 0.9	8.2 $\pm$ 0.3	29.8 $\pm$ 0.3	0.8 $\pm$ 0.1	0.4	$\beta + \alpha_2$
Swine....	4	4	15.2 $\pm$ 0.5	13.9 $\pm$ 0.2	8.2 $\pm$ 0.2		16.3 $\pm$ 0.6	6.0 $\pm$ 0.3	39.9 $\pm$ 0.8	0.4 $\pm$ 0.1	0.7	$\alpha_2$
Cow.....	4	5	11.0 $\pm$ 0.9	16.3 $\pm$ 0.5	13.7 $\pm$ 0.7		8.3 $\pm$ 0.3	10.7 $\pm$ 0.3	40.6 $\pm$ 1.6		0.7	$\beta$
Sheep....	5	6	15.0 $\pm$ 0.4	9.7 $\pm$ 0.2	15.0 $\pm$ 0.6		6.7 $\pm$ 0.4	9.8 $\pm$ 0.4	43.7 $\pm$ 0.7		0.8	"
Goat.....	1	7	12.9	7.6	3.9		12.7	13.7	49.2		1.0	$\alpha_2$
Dog.....	4	8	9.3 $\pm$ 0.6	13.3 $\pm$ 0.3	13.0 $\pm$ 1.6		8.0 $\pm$ 0.6	16.9 $\pm$ 0.3	39.6 $\pm$ 2.1		0.7	$\beta + \alpha_2$
Fox.....	4	9	3.9 $\pm$ 0.1	31.2† $\pm$ 1.9			7.8 $\pm$ 0.7	10.2 $\pm$ 1.1	47.1 $\pm$ 1.3		0.9	"
Cat.....	4	10	12.5 $\pm$ 1.1	5.2 $\pm$ 0.2	8.7 $\pm$ 0.5	4.7	20.2 $\pm$ 0.3	8.1 $\pm$ 0.4	41.4 $\pm$ 1.3		0.7	" + $\alpha_2 + \alpha_3$ , mostly $\alpha_3$
Mink.....	2	11	12.1	5.0	10.3	$\pm$ 0.6	9.8	11.7	51.5		1.0	$\beta + \alpha_2$
Rabbit...	5	12	4.3 $\pm$ 0.3	7.9 $\pm$ 0.8	13.0 $\pm$ 0.7			11.5 $\pm$ 0.8	63.3 $\pm$ 1.1		1.7	$\alpha$
Guinea pig.....	3	13	5.6 $\pm$ 0.3	8.1 $\pm$ 0.4	8.8 $\pm$ 0.1	15.2	3.7 $\pm$ 0.2	4.0 $\pm$ 0.4	54.6 $\pm$ 0.9		1.2	$\alpha_3$
Rat.....	4	14	4.8 $\pm$ 0.2	19.4† $\pm$ 0.5		$\pm$ 0.5		15.4 $\pm$ 0.6	50.1 $\pm$ 1.0	1.3 $\pm$ 0.1	1.4	$\beta + \alpha$
Chicken												
♂.....	4	15	37.5† $\pm$ 1.3				7.7 $\pm$ 0.5	15.8 $\pm$ 0.6	38.2 $\pm$ 1.3	0.5 $\pm$ 0.2	0.6	" + $\alpha_2$
Chicken												
♀.....	3		32.8† $\pm$ 1.2				11.3 $\pm$ 0.1	14.9 $\pm$ 1.0	40.4 $\pm$ 0.6	0.6 $\pm$ 0.1	0.7	" + $\alpha_2$

						$\beta + \alpha_2$ mostly				
						$\alpha_2$	$\beta + \alpha_2$	" + " mostly	$\alpha_2$	$\beta + \alpha_2$
Turkey...	4	16	8.1 $\pm$ 0.6	21.0† $\pm$ 0.7	14.1	4.3 $\pm$ 0.1	13.4 $\pm$ 0.4	51.5 $\pm$ 1.0	1.0 $\pm$ 0.1	1.1
Pheasant.	4	17	4.3 $\pm$ 0.4	16.3† $\pm$ 0.9		6.5 $\pm$ 0.3	14.0 $\pm$ 1.5	58.5 $\pm$ 1.4	0.4 $\pm$ 0.1	1.4
	4	18	7.7 $\pm$ 0.9	17.4† $\pm$ 1.2		4.5 $\pm$ 0.1	7.2 $\pm$ 0.3	64.1 $\pm$ 1.5	3.1 $\pm$ 0.6	1.8
Pigeon...	4	19	6.0 $\pm$ 0.3	15.5† $\pm$ 0.8		6.1 $\pm$ 0.4	21.9 $\pm$ 0.3	47.8 $\pm$ 0.7	2.6 $\pm$ 0.2	1.0
	3	20	2.1	7.1	14.1		6.8	67.7	2.1	2.1
Duck.....	1									
Carp.....										

of  $n$  observations,  $\lambda = \pm \frac{\sum(d)}{n\sqrt{n}}$ .

\* Average deviation of  $n$  observations,  $\Delta = \pm \frac{\sum(d)}{n\sqrt{n}}$ .

† Value for  $\phi + \beta$ .

‡ Value for  $\phi + \gamma + \beta$ .



The electrophoresis experiments were carried out in a modified (1) Tiselius apparatus, with a long, single section cell of 11 ml. capacity. A constant potential gradient of between 5 and 6 volts per cm. and a temperature of  $1^{\circ}$  were maintained. The duration of the electrophoresis experiment varied from 7200 to 10,800 seconds, the experiment being discontinued when the albumin (or component which is designated as *f*) on the ascending side had reached the end of the electrophoresis cell.

TABLE II

*Mobilities of Plasma of Various Species*

All mobilities are expressed as  $1 \times 10^{-5}$  sq. cm. per volt per second.

Species	$\gamma$	$\phi$	$\beta$	$\alpha_2$	$\alpha_2$	$\alpha_1$	Albumin	<i>f</i>
Man.....	1.3	2.3	3.1		4.3	5.4	6.6	
Monkey.....	1.4	2.2	3.3	4.2	4.8	5.4	6.6	7.4
Horse.....	1.4	2.5	3.7		4.8	5.8	7.1	7.9
Swine.....	1.8	2.8	3.7		4.4	5.2	6.5	7.3
Cow.....	1.4	2.6	3.7		4.7	5.5	7.0	
Sheep.....	1.6	2.6	3.4		4.3	5.3	6.8	
Goat.....	1.5	2.5	3.1		4.1	5.6	7.3	
Dog.....	1.2	2.4	3.3		4.4	5.7	6.8	
Fox.....	1.3	3.2*			5.2	6.4	7.5	
Cat.....	1.6	2.6	3.4	4.1	5.2	6.3	7.7	
Mink.....	2.2	3.2	3.7		3.8	4.2	4.9	
Rabbit.....	1.4	2.4	3.5			5.1	6.8	
Guinea pig.....	1.0	2.1	3.0	4.4	4.9	5.4	6.1	
Rat.....	1.6	2.7*			†		6.1	7.1
Chicken ♂.....	2.9†				4.6	6.1	7.3	8.1
"    ♀.....	2.5†				4.1	5.4	6.8	7.5
Turkey.....	1.7	2.9*			4.1	5.0	5.9	6.7
Pheasant.....	1.7	2.9*			3.6	4.2	5.2	6.1
Pigeon.....	1.7	3.3*			4.5	5.2	6.4	7.8
Duck.....	2.5	3.7*			4.9	5.8	6.7	7.6
Carp.....	1.8	2.6	3.4			4.0	6.8	

\* Value for  $\phi + \beta$ .

† Area too broad for mobility to be of significance.

‡ Value for  $\gamma + \phi + \beta$ .

The cylindrical lens sehlieren method as modified by Svensson (2) in conjunction with a diagonal knife-edge was used to photograph the moving boundaries. After enlargement, projection, and tracing, the areas under the curves were measured with a planimeter and the percentage composition was calculated from these figures. This calculation as well as that of the mobilities of the different proteins was made from measurements of the descending sides only, the net movement of any given component being

obtained by measuring the distance from the center of the salt boundary to the center of the area of the peak in question. The average percentage composition and mobilities of the plasma of all species studied are shown in Tables I and II respectively. Included in Table I is the plasma protein component with which the turbidity moved. The components of the plasma with which the turbidity moved were determined by qualitative observation of the light transmitted throughout the schlieren diagram.

Results of individual experiments for the various animal species are given below. In describing components we have used the usual nomenclature. In certain species, small amounts of a component appeared on the fast shoulder of the albumin peak and is designated *f*. While false boundaries due to convection currents appear more frequently just ahead of the albumin peak than anywhere else in the cell and might explain such a boundary, this does not appear to be the case here. The appearance of the *f* compo-

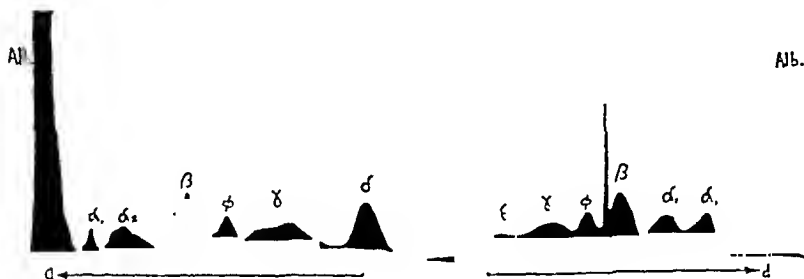


FIG. 1. Electrophoretic diagram of human plasma

nent was consistent in a given species, and, moreover, did not disappear when the plasma was studied at lower voltages than those usually used. This area has been considered as albumin in calculating the albumin-globulin ratios. Since human plasma has been studied rather extensively, it will be used as the basis for comparison of other plasmas.

*Human*—The usual six electrophoretic components including a descending  $\beta$  anomaly as recorded by various investigators (1-5) for human plasma are seen in Fig. 1. The results of three experiments shown in Table I give averages for these constituents that are compatible with values reported by Longworth (1), Dole and Braun (4), and Perlmann and Kaufman (5) for human plasma in barbiturate buffer, pH 8.6.

*Monkey*—The plasmas of three rhesus monkeys were analyzed. As shown in Fig. 2 there is a marked resemblance to human plasma, although three  $\alpha$ -globulins appear and a small amount of material moving faster than albumin is present. Analysis of these patterns reveals a somewhat lowered

amount of albumin compared with humans and slightly higher fibrinogen and  $\beta$ -globulin areas.

*Horse*—The blood used was obtained from aged horses at the time of slaughter. Previous electrophoretic analyses of horse serum have been reported by Svensson (6), van der Scheer *et al.* (7), and Tiselius and Kabat (8) under pH conditions which do not allow an easy comparison with our results at pH 8.6. Longworth (1) has indicated that a more satisfactory pattern for horse plasma is obtained with a phosphate buffer than with a barbiturate system. In Fig. 3 a typical electrophoretic pattern is presented.

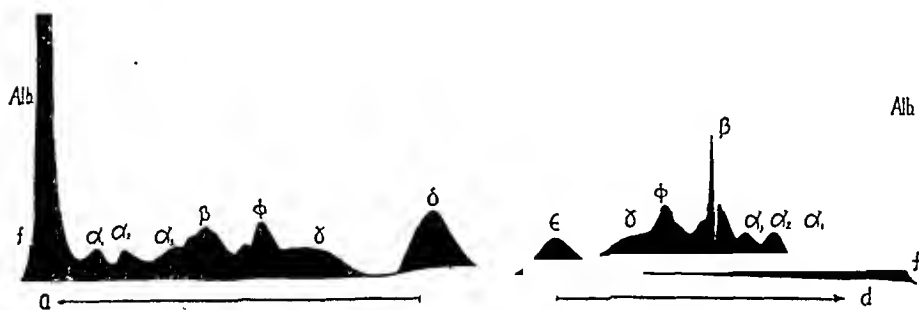


FIG. 2. Electrophoretic diagram of monkey plasma

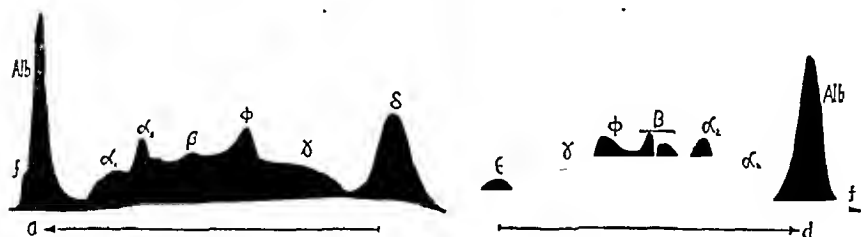


FIG. 3. Electrophoretic diagram of horse plasma

No distinct separation of  $\gamma$ -globulin and fibrinogen is seen. Chemical analyses indicate that fibrinogen constitutes 5 to 6 per cent of the horse plasma proteins (9, 10). Hence the area we have designated as fibrinogen may consist of another or of several other proteins, very likely analogous to the T component of van der Scheer *et al.* (7), an entity shown to be especially high in hyperimmune horse serum. Horse plasma shows evidences of the separation of two  $\beta$ -globulin components, although no  $\beta$  anomaly similar to that found in human plasma is evident. The opalescence was found to move with the  $\beta$ -globulin area in agreement with the observation of Svensson (6). Two distinct  $\alpha$ -globulins are present and are well separated from the albumin peak. The appearance of the  $f$  component, while

suggestive of a convection disturbance, appeared consistently in the plasma of this species. An extremely low albumin-globulin ratio further characterizes this plasma.

*Swine*—The blood of individual swine was obtained at the time of slaughter. A typical electrophoretic diagram is shown in Fig. 4. In this species  $\gamma$ -globulin is present in rather large amount and appears to be somewhat heterogeneous. The characteristic tall peak of fibrinogen lies in close proximity to that of the  $\beta$ -globulin and its large area suggests the presence of an additional protein or proteins having the same mobility. Chemical analyses indicate that approximately 8 per cent of the plasma protein of pigs is fibrinogen (10). A typical anomaly on the descending side is present in the  $\beta$ -globulin peak. The  $\alpha$ -globulin present is separated into two distinct portions, a small  $\alpha_1$  and a slower moving  $\alpha_2$  area present in a noticeably larger amount, 2 to 3 times that of the faster moving  $\alpha_1$  component. It was

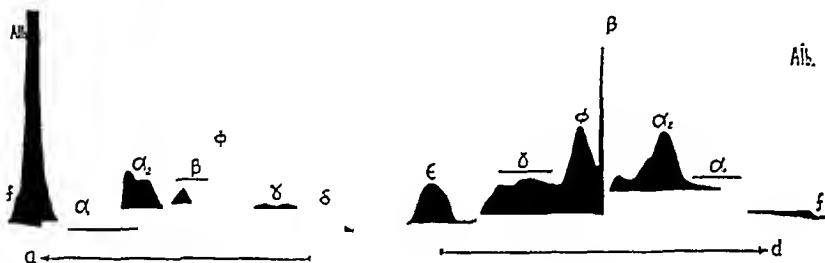


FIG. 4. Electrophoretic diagram of swine plasma

found that the plasma turbidity migrated with the  $\alpha$ -globulin in agreement with the findings of Svensson. Our results obtained from these experiments differ somewhat from those obtained by Svensson for serum in phosphate buffer, pH 7.7 (6), as is to be expected.

*Cow*—Blood was obtained from a slaughter-house. The plasmas showed relatively poor resolution for the slower globulins. Thus no clear separation of  $\gamma$ -globulin and fibrinogen is evident. Chemical analyses of bovine plasma for fibrinogen (9, 11) show considerable variation but indicate a level of from 3 to 10 per cent of the plasma protein. The high level of fibrinogen indicated by our electrophoretic analysis suggests that an additional protein or proteins may move in this area. The character of the area in Fig. 5 usually designated  $\gamma$ -globulin gives evidence that some faster  $\gamma$ -globulin may extend into this area and be partially responsible for the apparently high fibrinogen level. Two  $\alpha$ -globulins, rather closely associated, are present in approximately equal amounts and separate distinctly from the albumin.

*Sheep*—As indicated by Fig. 6, sheep plasma shows very poor resolution of components and the analysis for percentage composition of the several protein constituents is correspondingly difficult. The percentages and mobilities shown for the components in Table I represent an arbitrary analysis at best. The only component which has been evaluated with accuracy is the albumin. The  $\gamma$ -globulin appears to consist of two components, the faster of which has a mobility in close proximity with that of the fibrinogen. Chemical analyses of sheep plasma show the latter protein to constitute approximately 6 per cent of the plasma proteins (9, 10). Poor

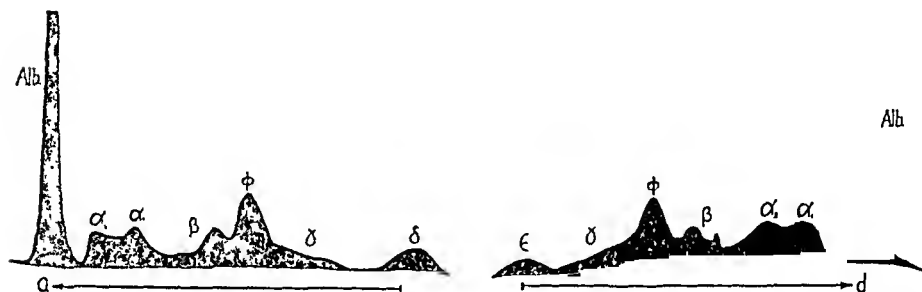


FIG. 5. Electrophoretic diagram of cow plasma

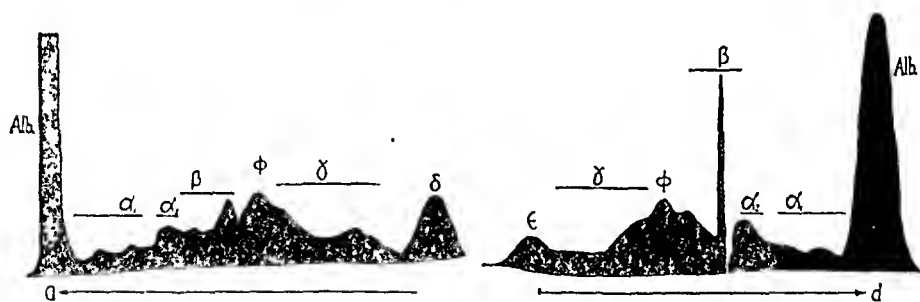


FIG. 6. Electrophoretic diagram of sheep plasma

resolution under the conditions of our experiments makes impossible, however, a good electrophoretic determination of the fibrinogen. The  $\beta$ -globulin area appears to consist of two poorly defined proteins, in the faster of which an anomaly of varying intensity appears. The slower moving portion of this  $\beta$ -globulin area is not separated from the fibrinogen. Perhaps the faster moving  $\beta$ -globulin protein better be classified as an  $\alpha$ -globulin. The data given in Table I should be considered with due regard to these facts.

*Goat*—The animal studied was a female Toggenburg. As shown in Fig. 7, the plasma protein of this animal resolves well under the experimental

conditions. The amount of fibrinogen found and recorded agrees well with the value reported for chemical analyses (12). A small peak analogous in mobility to  $\beta$ -globulin appears on the fast side of the fibrinogen on the descending side and as a more distinct entity on the ascending limb of the pattern. The turbidity moves with the next component which we have designated  $\alpha_2$ -globulin on the basis of its mobility. An anomaly appears with this peak. Such designation results in a very high percentage of the plasma proteins of the goat as  $\alpha$ -globulin entities.

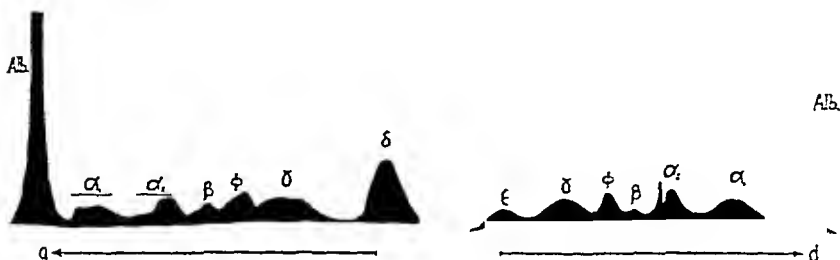


FIG. 7. Electrophoretic diagram of goat plasma

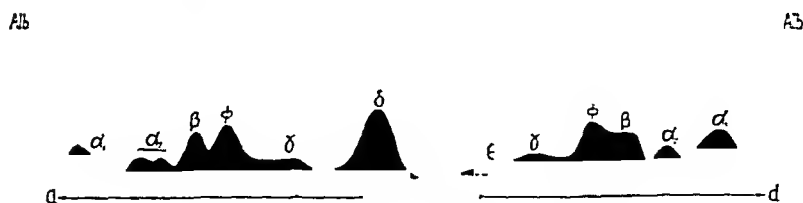


FIG. 8. Electrophoretic diagram of dog plasma

*Dog*—The electrophoretic pattern of Fig. 8 for dog plasma shows a close resemblance in separation and mobility of components to that of humans. However, good separations of  $\gamma$ -globulin, fibrinogen, and  $\beta$ -globulin did not occur on the descending side, in agreement with the recent work of Zeldis and Alling (13). However, in contrast to these authors, we observed no  $\beta$  anomaly. As compared to the variable results of chemical analysis for fibrinogen, which indicate from 4 to 10 per cent fibrinogen in dog plasma (10, 14, 15), the relatively higher level of fibrinogen evidenced in this work suggests the presence of an additional component in this area. Such a protein would be compared to the so called T component of horse serum (7). Two relatively unsymmetrical  $\alpha$ -globulin boundaries are present. The  $\alpha_2$ -globulin shows separation into two components on the ascending side.

The  $\alpha_1$ -globulin is relatively high in amount as compared to human plasma, while the reverse is true for albumin. The analytical data of Zeldis and Alling (13), while showing more individual variation of all components and higher albumin values, are in essential agreement with our work. Although our  $\alpha$ -globulin areas were designated  $\alpha_1$  and  $\alpha_2$ , they were not homogeneous and, particularly on the ascending side, might have been divided into as many as four  $\alpha$ -globulins, as was done in some of the analyses of Zeldis and Alling (13).

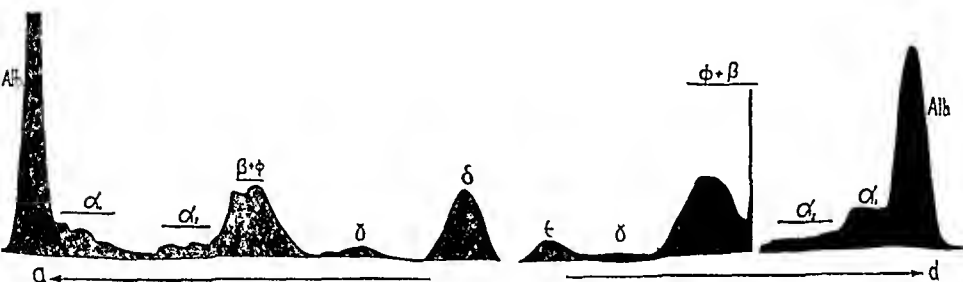


FIG. 9. Electrophoretic diagram of fox plasma

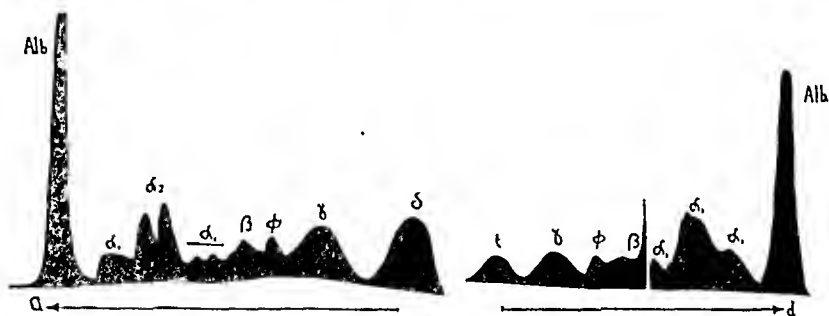


FIG. 10. Electrophoretic diagram of cat plasma

*Fox*—The animals used were ranch-raised silver fox. As is seen from Fig. 9, their plasma is characterized by a low  $\gamma$ -globulin content, failure of the fibrinogen and  $\beta$ -globulin components to resolve, and the presence of a broad, irregular  $\alpha$ -globulin area. A characteristic  $\beta$  anomaly was present on the descending side. A broad  $\alpha$ -globulin area, while not showing good resolution, was calculated as an  $\alpha_1$  and an  $\alpha_2$  component. The corresponding ascending area was extremely complex.

*Cat*—It appears from Fig. 10 that cat plasma is characterized by a multiplicity of components that show good separation. The  $\beta$ -globulin possesses an anomaly on the descending side. The area immediately following this

component, although rather closely associated with this anomaly, is analogous in position and mobility to human  $\alpha_2$ -globulin. On the ascending side this component separates into a distinct heterogeneous area and for this reason has been arbitrarily termed an  $\alpha$ - rather than a  $\beta$ -globulin. Beyond this region are two further  $\alpha$ -globulin areas, a relatively large one showing distinct separation into two components and a smaller following one. This latter area, in contrast to analogous  $\alpha_1$ -globulins of most other species, lies at a considerable distance from the albumin. The faster components,

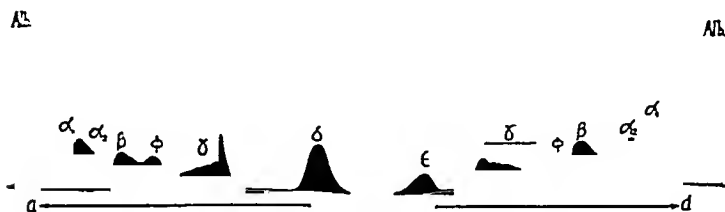


FIG. 11. Electrophoretic diagram of mink plasma

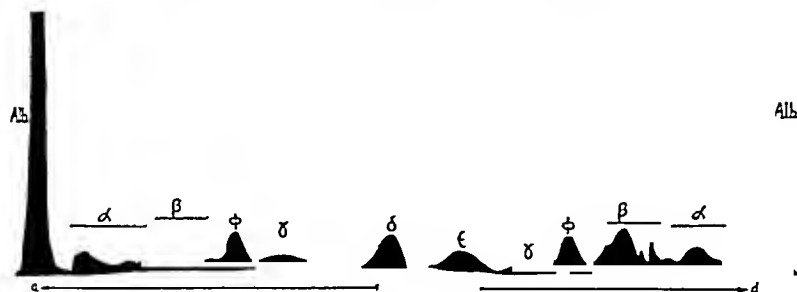


FIG. 12. Electrophoretic diagram of rabbit plasma

particularly the albumin, possess mobilities which are considerably higher than those for the corresponding human components.

*Mink*—Blood was obtained from ranch-raised mink at pelting time. Fig. 11 indicates that their plasma is characterized chiefly by a disturbance in the  $\gamma$ -globulin area. This globulin peak is very irregular in shape. The remaining constituents, while not too well resolved on the descending side, do appear as distinct entities. The area we have designated as fibrinogen does not appear as the usual characteristic sharp peak and possibly the disturbance in the  $\gamma$ -globulin may be due to the fibrinogen. No anomaly is



present in the  $\beta$ -globulin area. The albumin possessed a mobility considerably lower than that of the other mammals studied.

*Rabbit*—Adult rabbits of mixed breed were the source of this plasma. Distinct separation of major components is indicated by Fig. 12. Our electrophoretic values for fibrinogen are approximately twice as large as the values obtained by chemical analysis.<sup>2</sup> The  $\beta$ - and  $\alpha$ -globulin areas appear as relatively irregular zones with no marked anomaly present, although the turbidity appeared to migrate with the slowest of the  $\alpha$ -globulins. The concentration of the  $\alpha$ -globulins is relatively low. As a result of the high albumin content, an albumin-globulin ratio distinctly above that of man is seen. This is compatible with the work of Svensson (6) on rabbit sera, although his experiments were carried out under different conditions and do not lend themselves readily to direct comparison.

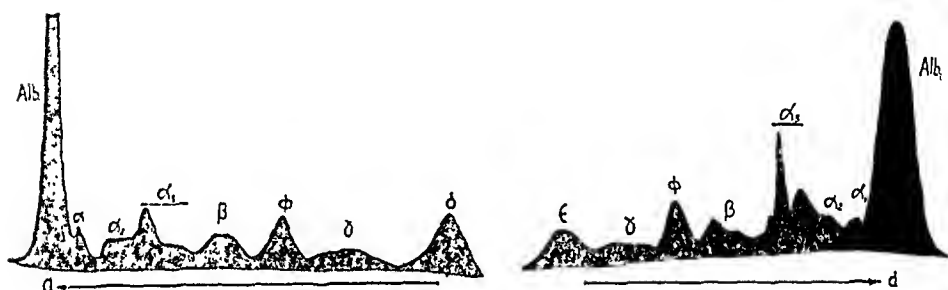


FIG. 13. Electrophoretic diagram of guinea pig plasma

*Guinea Pig*—Plasma was obtained from adult, female, albino guinea pigs. Distinct differences from human plasma are seen in Fig. 13. An anomaly analogous to that seen in the  $\beta$ -globulin fraction of human plasma and serum appears in the descending component which migrates with the velocity of human  $\alpha_2$ -globulin. This area is unsymmetrical and is approximately twice as large as the corresponding  $\alpha_2$ -globulin peak in human plasma. The area corresponding to human  $\alpha_1$ -globulin appears to consist of two proteins. In fact, all of the constituents, with the exception of the fibrinogen, are relatively heterogeneous. The chemical analyses of Howe (9) and Link<sup>2</sup> and our electrophoretic data for the latter entity of guinea pig plasma are in good agreement. An albumin-globulin ratio distinctly lower than that of humans is seen.

*Rat*—Adult, breeding, albino male rats of the Sprague-Dawley strain were the source of plasma. Fig. 14 shows that very poor separation of components was effected on electrophoresis. The descending pattern suggests that the usually rather sharp fibrinogen component is moving with a rela-

<sup>2</sup> Link, K. P., personal communication.

tively broad  $\beta$ -globulin area. An anomaly is seen at the junction of the  $\beta$ - and  $\alpha$ -globulins on this side. It is impossible to determine the fibrinogen concentration from our patterns. Since chemical analyses by Kline (16) show that approximately 4 per cent of rat plasma protein is fibrinogen, the  $\beta$ -globulin area comprises about 15 per cent of these proteins. No separation into distinct components of a broad area corresponding to the  $\alpha$ -globulins is evidenced. Li (17) and Moore *et al.* (18) found five components separating in normal rat sera and, in contrast to our work, found that the  $\alpha$ -globulin area resolved itself into two distinct components. The albumin-



FIG. 14. Electrophoretic diagram of rat plasma

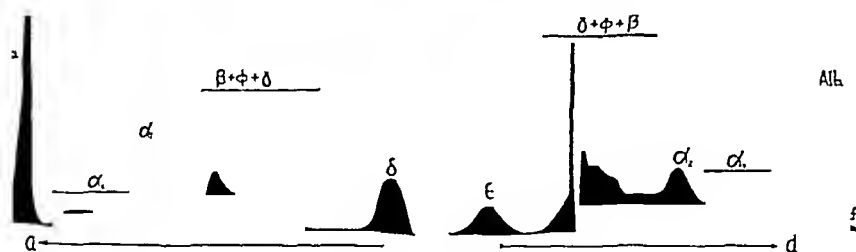


FIG. 15. Electrophoretic diagram of chicken plasma

globulin ratios given by Moore *et al.* at pH 8.5 are considerably higher than those found in our work. These variations may be due to differences in the age of the animals used. The  $f$  component was present in all of the rat plasmas studied.

*Chicken*—The animals used were adult white Leghorns which had been maintained under range conditions. Plasmas from both males and females were analyzed. A typical plasma pattern of a female is shown in Fig. 15. A slight difference between male and female plasmas was apparent. Slight differences in percentages and distinct differences in the mobilities of the plasma components of males and females are shown in Table I. The

marked anomaly appeared to be more closely associated with the  $\gamma$ -globulin in the case of the female and with the  $\beta$ -globulin in the case of the male. The relation of fibrinogen to these anomalies is speculative, since it did not separate as a distinct entity in any experiment, although it lies in the regions of the anomaly and might well be responsible for it. In the male, the  $\alpha_1$ -globulin appears as a more distinct entity than in the case of the female. Failure of separation of components in the  $\gamma$ -globulin-fibrinogen region makes difficult the analysis of these patterns.

Other electrophoretic work on chicken plasma has been reported and the disturbance of the kind noted above has been described. Sanders *et al.* (19) state that a pronounced  $\gamma$ -globulin disturbance is present in the patterns of all normal chicken sera and plasma, although their photograph of normal plasma shows the disturbance to be definitely located in the  $\beta$ -globulin area. They obtained good separation of fibrinogen in their work in contrast to our findings. The anomaly present in serum of chickens

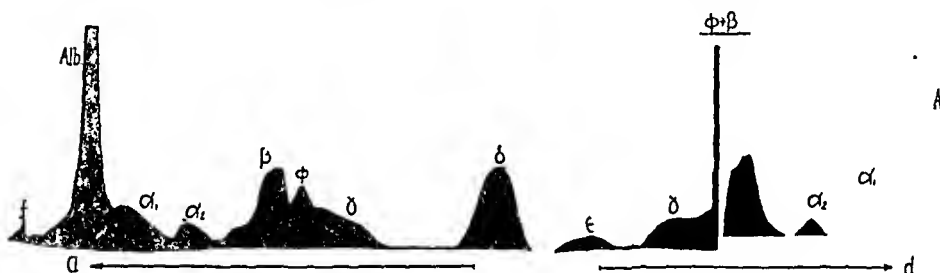


FIG. 16. Electrophoretic diagram of turkey plasma

afflicted with leucosis and referred to as the *L* component by Sanders *et al.* (19) appears to be similar to the disturbance present in our plasmas. Such an anomaly may represent the chicken's normal response to foreign antigen and may have resulted in our case from the usual infections met with in chickens under range conditions.

*Turkey*—The animals used were bronze turkeys, approximately three-quarters grown. As is seen in Fig. 16, the electrophoretic diagrams are similar to those of other fowls studied in that there is a failure of  $\gamma$ -globulin, fibrinogen, and  $\beta$ -globulin to resolve on the descending side. An anomaly in this region appears in the area usually occupied by fibrinogen. The ascending side, however, shows evidence of separation of these components. The presence of two  $\alpha$ -globulins is demonstrated. The peak of the  $\alpha_1$  component was not symmetrical and was poorly separated from the albumin. A small amount of material moving faster than albumin was also present. This is characteristic of the electrophoretic patterns of the other fowls studied.

*Pheasant*—The animals were ring-necked pheasants that had been raised in pens. From Fig. 17 it is seen that their plasma diagrams possess characteristics that resemble in general the patterns for the other fowls studied. Separation of fibrinogen and  $\beta$ -globulin occurs only on the ascending side. An electrophoretic analysis of serum showed a decrease of 9 per cent in the peak of the  $\beta$ -globulin-fibrinogen complex on the descending side and the absence of a separate fibrinogen peak on the ascending limb. A distinct anomaly appears at the point where  $\gamma$ -globulin and fibrinogen separate in

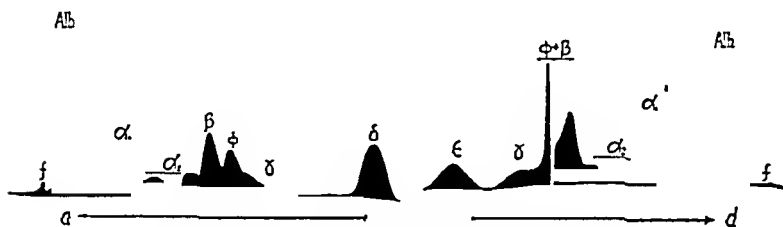


FIG. 17. Electrophoretic diagram of pheasant plasma

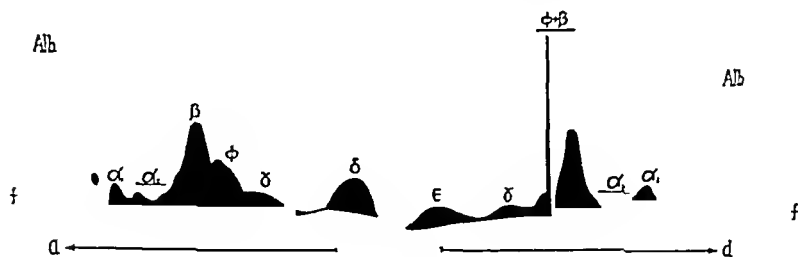


FIG. 18. Electrophoretic diagram of pigeon plasma

human plasma. There are evidences of three  $\alpha$ -globulins; the portion analogous to human  $\alpha_1$ -globulin is relatively large in amount, while the  $\alpha_2$  analogues are present in smaller quantities. There is a very small component migrating on the fast shoulder of the albumin peak. As is usually the case, this component appears as a more distinct entity on the ascending side.

*Pigeon*—Adult white king pigeons were used in this work. There was a failure of fibrinogen and  $\gamma$ -globulin to separate on the descending side. Furthermore a marked anomaly appeared in this area, a characteristic already noted in the diagrams for fowl plasma. A typical electrophoretic

pattern is shown in Fig. 18. Two poorly resolved  $\alpha$ -globulins are indicated. A high albumin-globulin ratio and a comparatively large amount of a component moving on the fast shoulder of the albumin peak are characteristics of the plasma of this animal.

*Duck*—The plasmas of three adult ducks were studied. One animal was a muscovy duck; the remaining two were first generation progeny of a muscovy-mallard cross. Fig. 19 shows that their plasmas are characterized by the very marked anomaly which is seen in the electrophoretic patterns for all of the birds studied. As indicated previously, the protein responsible for this anomaly, or associated with it, is not known. The assignment of designations to the components in Fig. 19 is necessarily arbitrary. It is likely that the fibrinogen is represented by the large peak following this anomaly which has been designated the fibrinogen- $\beta$ -globulin area. A sample of duck plasma which had partially clotted showed a decrease in the

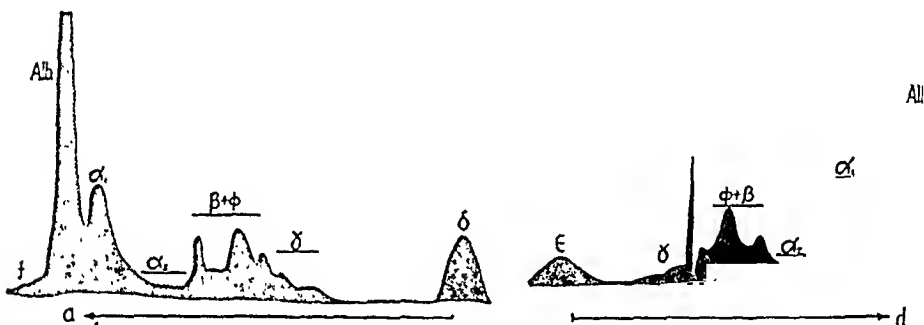


FIG. 19. Electrophoretic diagram of duck plasma

higher peak of this area. The turbidity of the plasma migrated on the fast side of these proteins at the junction of the  $\phi + \beta$  and  $\alpha_2$  areas. The association of the  $\beta$ -globulin and lipid of serum and plasma is characteristic (20), although it is not known whether it is responsible for the  $\beta$  anomaly of human plasma. Interesting enough is the presence of an anomaly and the evidence for the lipid being carried by another component of the fowl plasma. A large amount of  $\alpha_1$ -globulin which separates rather poorly from the albumin further characterizes duck plasma. A small amount of a component migrating with a greater velocity than albumin is also present. The components, particularly the  $\gamma$ -globulin, have unusually high mobilities.

*Carp*—It was found to be extremely difficult to obtain suitable samples of carp plasma or serum because of hemolysis. Consequently, only one determination was made on this animal (*Cyprinus carpio*). As shown in Fig. 20, the electrophoretic pattern of this animal's plasma shows extreme difference from that of man or of the other species studied. A small area corre-

sponding to the  $\gamma$ -globulin is followed by an irregular portion which in the plasma pattern of humans represents fibrinogen. Two areas which we have designated as  $\beta$ - and  $\alpha$ -globulin follow. The turbidity in this plasma moves with the peak designated  $\alpha$ -globulin. The very large albumin area shows the beginning of a resolution into two peaks. Strangely enough this separation is better on the descending than on the ascending side. It is possible that the slower moving portion may be analogous to  $\alpha_1$ -globulin of humans, although the mobility of this whole area is analogous to human  $\alpha_1$ -globulin rather than to the albumin. A considerable amount of material moving faster than albumin is also present. The relatively high albumin-globulin ratio agrees with the chemical data of Field *et al.* (21), if allowance is made for the fact that ratios determined electrophoretically at pH 8.6 have been shown to approximate two-thirds of the ratios obtained by salt fractionation (4). Field *et al.* (21) report a plasma fibrinogen level of 5.5 per cent.



FIG. 20. Electrophoretic diagram of carp plasma

Our fibrinogen area is somewhat larger, but the complexity of the pattern suggests that several proteins are present here.

#### DISCUSSION

From the electrophoretic analysis of the various plasmas it is obvious that a great deal of variation in the distribution of the blood proteins occurs among different species of animals. This variation appears as differences in mobilities, in the number and relative proportions of components, and in the failure of some plasmas to show good resolution of components.

In many of the plasmas studied it is impossible to ascertain the amount of particular globulins present, owing primarily to the fact that they are not sufficiently separated. In other words, differences in their mobility in an electrical field are too small for good resolution. This was often true in the case of fibrinogen, in which evidences of this protein's presence were sometimes seen in the form of a sharp peak superimposed on a mass of other protein. In some species fibrinogen peaks separated well but their percent-

age of the total protein was considerably higher than that expected from the chemical analysis reported for the same species. This indicates the presence of a protein or proteins migrating with fibrinogen, and hence fibrinogen values reported in this work should be necessarily looked upon as collective values for proteins possessing the mobility of fibrinogen. It would be desirable to examine serum as well as plasma in individual species in order to make a more exact study of these relationships.

All of the fowl plasmas studied were characterized by diagrams with a serious disturbance of the kind described as the  $\beta$ -globulin anomaly of humans. This anomaly, however, was present in the fraction corresponding to either human  $\gamma$ -globulin or a protein of slightly higher mobility. Since the turbidity moved with faster migrating proteins, they did not appear to be responsible for the anomaly. In chickens the anomaly appeared to vary slightly, being more intimately associated with the  $\gamma$ -globulin in the plasma of females, while in males it was associated with a somewhat faster migrating protein. The anomaly does not appear to be due to fibrinogen, since a pheasant serum retained the anomaly typical of plasma and the same was noted for a sample of duck plasma that had partially clotted. The nature and association of this disturbance are at present unknown, although it tends to make difficult the analysis for the amounts of the proteins having mobilities in this area.

A good deal of variation in the number of components was noted from animal to animal. Most of this variation was usually experienced in the region of electrophoretic mobility which is usually ascribed to the  $\alpha$ -globulins. These  $\alpha$ -globulins, or at least components resembling them in mobility, often were concerned with the lipid present in the plasma, as is indicated by the movement of turbidity with these proteins. However, in many animals the turbidity which is very probably associated with lipid appears to be carried by several plasma protein components. In the case of the fowl plasmas studied this turbidity migrated on the fast side of the  $\phi + \beta$  complex and seemed to be associated predominantly with the  $\alpha_2$ -globulin. The marked anomaly observed in the  $\phi + \beta$  complex of the fowl plasmas did not appear to be associated with the turbidity. In humans the turbidity is associated with the  $\beta$  anomaly as well as with the  $\alpha_2$ -globulins.

As is seen from Fig. 1, the association of plasma turbidity in the case of some species was entirely with the  $\alpha$ -globulins. In general, the slower  $\alpha$ -globulin component of the plasma appeared to carry a good deal of the lipid, although in some cases the  $\beta$ -globulin was entirely responsible. The designation of the lipid-carrying  $\alpha$ -globulins as such is arbitrary and they might equally well be considered as  $\beta$ -globulins of high mobility.

Much variation in the mobility of plasma components rather easily recognized, such as  $\gamma$ -globulin and albumin, was apparent. This is not

surprising, since Landsteiner, Longworth, and van der Schuer (22) have shown that there is also a variation in the electrophoretic mobilities of hemoglobin and of egg albumin of different species. Such differences appear in the other blood proteins as well, although Tiselius and Kabat (8) have reported that the mobilities of the various normal components in sera from different animals were much the same.

Certain of the species studied, particularly the fowl, showed the presence of a small amount of protein which migrated with a mobility somewhat higher than that of albumin. In certain species, such as the horse (see Fig. 3), this component was suggestive of convection in the cell but the regularity of its appearance even when the voltage was reduced below that normally used did not favor such a possibility. As was to be expected, these proteins were seen as more distinct entities on the ascending than the descending side of the diagram. In addition, the presence of very small amounts of protein migrating with high velocity, 2 to 3 times as great as that of albumin, was often noted early in the experiment. These latter fast components did not seem to occur constantly in a given species, but appeared in various plasmas in a random fashion. Their relation to the plasma proteins is not known.

Differences in albumin-globulin ratios were likewise noted among the different species. The work of Dole and Braun (4) indicated that albumin-globulin ratios of humans determined electrophoretically at pH 8.6 usually approximated two-thirds of the ratios determined by salt fractionation methods. Our work is in agreement, since our albumin-globulin ratios are correspondingly less than values which have been obtained by other workers using the salt fractionation methods (23). The work of Zeldis and Alling (13) on dogs shows that the albumin-globulin ratio determined electrophoretically is approximately four-tenths of the ratio as determined chemically. This difference from the human points to the likelihood of variation of such ratios from species to species.

The work presented here for individual species is based on a relatively small number of animals. It has sufficed, however, to show the approximate electrophoretic patterns characteristic of the species examined and to point out the manifold differences which may be encountered.

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## SUMMARY

The electrophoretic patterns of plasma from twenty species of animals obtained under identical conditions reveal distinct species variations in mobility, amount, and number of protein components. In a given species, however, the analytical data are relatively constant in regard to the above criteria.

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# SPECIES DIFFERENCES IN SERUM PROTEIN PATTERNS

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Electrophoretic patterns of the serum of several normal animal species have been published (1-19). These patterns, however, have not lent themselves to a direct comparison with each other and it has been generally assumed that the sera of various species do not differ greatly, except that most sera of normal rabbits (13) and rats (16) do not contain the usual  $\alpha$ -globulin. Bourdillon and Lennette (4) have obtained patterns on mouse sera in phosphate buffer at pH 7.5 which indicated the presence of two components in the  $\alpha$ -globulin region and small amounts of  $\beta$ - and  $\gamma$ -globulins. Pillemer *et al.* (20) analyzed guinea pig serum but published no patterns.

During the past 5 years the sera from a variety of animal species have been studied in this laboratory and it has been observed that there are appreciable and characteristic specific differences in the electrophoretic patterns obtained under comparable conditions. The characteristic patterns of each species are highly reproducible and the differences in respect to both quantity and presence or absence of components is significant.

## Method

The blood samples were taken in every case from living animals. The smaller animals were bled from the heart, usually under ether anesthesia. Venous blood from man and the larger animals was used. The clear serum was collected from the clotted and centrifuged blood and examined electrophoretically after dilution with and dialysis in the cold against the various buffers. Most of the analyses were made either in a 0.02 M sodium phosphate buffer containing 0.15 M NaCl at pH 7.4 or a barbiturate buffer containing 0.1 M sodium diethyl barbiturate and 0.02 M diethylbarbituric acid at pH 8.6, as described by Longsworth (15). A few analyses were made in the phosphate-NaCl buffer at different pH values and in a barbiturate-LiCl buffer at pH 7.8 (3).

The type of pattern obtained depends appreciably on the buffer used and patterns cannot be compared unless the conditions of the experiments are similar. The  $\gamma$ -globulin does not separate from the salt or  $\epsilon$  boundary (21) in either the phosphate buffer or the barbiturate-LiCl buffer at pH 7.8. Consequently the percentage composition of this slowest moving component is erroneously large by an amount dependent on the serum dilution (22).

In the barbiturate buffer at pH 8.6 the  $\gamma$ -globulin separates from the salt boundary so that it is possible to determine its magnitude more accurately, but in this buffer the  $\beta$ - and  $\gamma$ -globulins do not separate as sharply and the components are sometimes not as well defined as in phosphate at pH 7.4.

### Results

A summary of the electrophoretic partitions of normal sera is presented in Table I and typical patterns obtained in phosphate-NaCl buffer at pH 7.4 and barbiturate buffer at pH 8.6 for each species are shown in Figs. 1 and 2 respectively. All data were taken from the descending patterns except for  $\beta$ -globulin in man and monkey, in which the  $\beta$  spike interfered with measurements. In these cases the ascending pattern for  $\beta$ -globulin was used. Whenever twelve or more patterns on the same species were obtained under similar conditions, mean values with standard deviations are included in Table I. The number of blood samples examined is given in the second column. Each sample was from a different animal except when the same sample was examined in two buffers. Since in many species it has not yet been determined which components are albumins and which are globulins, etc., the components have been designated in Table I by numbers from 1 to 6 in order of decreasing mobilities.

*Patterns in Phosphate-NaCl Buffer at pH 7.4*—Of the species studied in phosphate buffer at pH 7.4, sera from man, rhesus monkey (*Macaca mulatta*), and swine were found to be the most similar, each having one albumin and three globulin components (1), although the average ratio of albumin to globulin was considerably higher in human sera. The proportion of the components in the monkey serum was quite variable and it is possible that the serum from monkeys in their natural habitat would have a higher albumin-globulin ratio, since monkeys may not remain in prime condition after they are subjected to laboratory living conditions.

The swine serum from which the data of Line 12 (Table I) were calculated was obtained from a New York city slaughter-house, whereas the sera furnishing the data of Lines 13, 14, and 15 were obtained from 9 month-old swine raised privately in Dutchess County, New York. There was an agreement between the percentages of  $\gamma$ -globulin of the latter, whereas the former serum had a distinctly higher  $\gamma$ -globulin and the lowest albumin of all.

The dogs used were mostly medium sized mongrels. Their sera had an average albumin-globulin ratio of unity and a large  $\beta$ -globulin which usually separated into two components.

As has already been pointed out, rabbit (cross-breeds of Belgian hare, chinchilla, and New Zealand) and rat (Long-Evans strain) sera do not usually contain  $\alpha$ -globulin. The ratio of albumin to globulin of these two

species was the highest of any studied and the  $\gamma$ -globulin in many rats was found to be extremely low. In the sera from a series of young rats, 21 to 29 days old, it was found that the young rat sera, contrary to that of the adult, contained a definite  $\alpha$ -globulin and a hardly measurable amount of  $\gamma$ -globulin. The patterns for the young rats contained a spike at the position of the

TABLE I

*Electrophoretic Fractionation of Serum Proteins of Various Animal Species*

Line No.	No. of serum samples used	pH and buffer <sup>a</sup>	Ionic strength	Dilution <sup>b</sup>	Composition, per cent						Mobilities <sup>c</sup>					
					Component No.						Component No.					
					1	2	3	4	5	6	1	2	3	4	5	6
2 Man																
3	1	7.4 P.	0.2	1:4	64		10	16		10	4.7		3.2	2.3		0.5
4	1	7.4 "	0.2	1:4	66		8	15		11	5.4		3.8	2.7		0.9
5	25	7.8 B.	0.05	1:4	67		7	13		13	6.6		5.0	3.3		0.4
					±11		±1	±2		±2	±0.4		±0.3	±0.3		±0.3
6	15	8.6 "	0.1	1:3	63	5	7	13		12	5.9	5.1	4.1	2.8		1.0
					±3	±1	±1	±2		±3	±0.3	±0.2	±0.3	±0.2		±0.3
7 Monkey																
8	1	7.6 P.	0.2	1:4	61		3	20		16	5.5		4.0	3.2		1.5
9	1	7.6 "	0.2	1:4	47		11	13		29	5.8		4.0	3.2		1.5
10	1	7.6 "	0.2	1:4	48		11	15		26	5.0		3.6	2.8		1.4
11 Swine																
12	1	7.4 P.	0.2	1:4	40		16	19		25	4.9		3.4	2.8		1.4
13	1	7.4 "	0.2	1:4	46		21	14		19	4.3		3.0	2.2		0.6
14	1	7.4 "	0.2	1:4	53		20	11		16	4.5		3.1	2.4		0.9
15	1	7.4 "	0.2	1:4	49		17	16		18	4.9		3.4	2.4		1.2
16	1	8.6 B.	0.1	1:2	45	4	13	8	12	8	4.9	4.4	3.1	2.4	2.0	1.5
						10						3.9				
17 Dog																
18	17	7.4 P.	0.2	1:4	50		10	16	10	14	5.5		3.8	2.6	2.0	0.7
					±4		±4	±5	±7	±3	±0.3		±0.3	±0.3	±0.3	±0.3
19 Rabbit																
20	1	7.5 P.	0.2	1:4	75			13		12	5.2			3.0		0.8
21	1	8.2 "	0.2	1:4	70		6	12		12	6.0		4.8	3.3		1.0
22	12	7.8 "	0.2	1:4	64			15		21	5.1		4.2	3.0		0.9
					±7			±3		±6	±0.2		±0.2	±0.2		±0.1
23	1	8.6 B.	0.1	1:3	65	7	7	11		10	6.7	5.1	4.2	3.2		1.3
24	1	8.6 "	0.1	1:3	63	3	8	13		14	6.0	5.5	5.0	3.1		1.2

TABLE I—Continued

Line No.	No. of serum samples used	pH and buffer*	Ionic strength	Dilution†	Composition, per cent						Mobilities‡					
					Component No.						Component No.					
					1	2	3	4	5	6	1	2	3	4	5	6
Rat																
25																
26	20	7.4 P.	0.2	1:3	72				17	11	4.7				2.2	0.9
					±5				±6	±3	±0.2				±0.2	±0.1
27	1	8.6 B.	0.1	1:3	58	20	2		15	5	5.9	5.1	4.7		2.9	1.8
28	1	7.4 P.	0.2	1:3	70		9		12	9	4.8		3.2		2.4	1.3
29	1	7.4 "	0.2	1:3	76		9		12	3	4.6		3.0		2.0	0.7
30	1	7.4 "	0.2	1:3	76		6		11	7	4.4		2.8		2.0	0.5
31	1	8.6 B.	0.1	1:3	72	6	8		11	3	5.9	5.5	4.0		2.7	1.5
Cotton-rat																
32																
33	1	7.4 P.	0.2	1:3	50		25		14	11	4.8		3.2		2.5	0.5
34	1	7.4 "	0.2	1:3	53		28		10	9	4.4		2.8		2.1	0.4
35	1	8.6 B.	0.1	1:3	43	10	28		12	7	6.2	5.1	4.0		2.9	0.8
36	1	8.6 "	0.1	1:3	51	5	30		7	7	6.3	4.9	4.1		3.0	1.1
Guinea pig																
37																
38	1	7.4 P.	0.2	1:3	67	15	4		5	9	4.4	3.6	3.1		1.8	0.6
39	1	7.4 "	0.2	1:3	71	14	5		3	7	4.1	3.7	3.1		1.7	0.6
40	1	7.4 "	0.2	1:3	56	16	5		11	12	4.1	3.5	3.0		1.7	0.7
41	15	8.6 B.	0.1	1:3	54	11	21		6	8	6.1	4.8	4.1		2.7	1.1
					±12	±2	±5		±2	±4	±0.6	±0.5	±0.2		±0.4	±0.2
Hamster																
42																
43	1	7.4 P.	0.2	1:2	54	12			7	10	17	4.3	3.4		2.6	1.5
44	1	7.4 "	0.2	1:3	65	15			5	8	7	4.5	3.8		3.0	1.8
45	1	6.6 "	0.2	1:2	60	15			4	9	12	4.0	3.2		2.2	1.7
46	1	7.4 "	0.2	1:3	56	13			8	14	9	4.4	3.5		2.5	1.7
47	1	7.4 "	0.2	1:3	66	13			5	9	7	4.3	3.5		2.6	1.5
48	1	8.6 B.	0.1	1:4	58	11	5	16	5	5	6.2	5.0	4.0	3.5	2.1	1.2
49	1	8.6 "	0.1	1:3	61	12	5	11	5	6	5.8	4.8	4.0	3.4	2.6	1.9
Cat																
50																
51	1	7.4 P.	0.2	1:4	35	5	13	14		33	6.5	5.3	4.5	3.1		1.0
52	1	7.4 "	0.2	1:4	29	10	13	15		34	6.7	5.6	4.7	3.4		1.4
53	1	7.4 "	0.2	1:4	32	6	14	19		29	6.9	6.0	4.9	3.6		1.4
54	1	8.6 B.	0.1	1:4	36	20		13		31	8.4	6.2		3.6		2.0
55	1	8.6 "	0.1	1:4	35	25		12		28	8.1	5.7		3.6		1.4
56	1	7.4 P.	0.2	1:3	43	14	15	21		7	5.7	4.7	3.9	3.3		0.8
57	1	7.4 "	0.2	1:3	40	15	17	17		11	6.2	5.0	4.1	3.0		1.0
58	1	8.6 B.	0.1	1:3	46	12	11	12		5	7.6	6.0	4.7	3.2		1.8
						14						5.3				

TABLE I—Concluded

Line No.	No of serum samples used	pH and buffer*	Ionic strength	Dilution†	Composition, per cent						Mobilities‡					
					Component No.						Component No.					
					1	2	3	4	5	6	1	2	3	4	5	6
59 Chicken, hen																
60	1	7.4 P.	0.2	1:4	7	38		8	16	31	6.2	5.3		3.3	2.0	1.3
61	1	7.4 "	0.2	1:4	4	26			40	30	5.4	4.7			1.3	1.0
62	1	7.4 "	0.2	1:4	3	27	7	13	27	22	5.8	4.9	4.2	3.3	2.0	1.3
63	1	7.4 "	0.2	1:4	5	25	4	8	43	15	6.8	5.4	4.5	3.3	2.3	1.3
64	1	7.4 "	0.2	1:4	6	36	8	6	27	17	6.0	5.0	4.0	2.8	1.6	1.0
65	1	6.5 "	0.2	1:4	7	20	3	7	54	9	4.4	4.1	3.5	2.7	1.4	0.8
66 Chicken, cockerel																
67	1	7.4 P.	0.2	1:3	47	13	11	14		15	5.3	4.4	3.2	2.3		1.4
68	1	7.4 "	0.2	1:3	45	17	10	8		10	5.1	4.2	3.3	2.2		1.5
69	1	7.4 "	0.2	1:3	39	16	9	19		17	5.1	4.1	3.2	2.4		1.7
70 Pigeon																
71	1	7.4 P.	0.2	1:3	57			19	16	8	4.1			2.2	1.6	0.3
72	1	8.6 B.	0.1	1:3	58	10	5		18	9	5.8	4.4	2.8		2.4	1.7
73 Fish, carp																
74	1	7.4 P.	0.2	1:3	48			16	13		23	4.2		3.4	2.5	0.9
75	1	8.6 B.	0.1	1:3	47			21	12		20	6.0		3.7	2.9	1.7

Line 5, data taken from reference (9). Line 6, data taken from reference (19). Line 22, data taken from reference (13). Lines 28 to 31, data on pooled sera of baby rats aged 21, 29, 21, 25 days, respectively. Line 33, data on three pooled sera. Lines 34, 35, 36, data on two pooled sera. Lines 56, 57, 58, data on sera of 4 day-old kitten.

\* P., sodium phosphate-NaCl buffer; B., barbiturate buffer.

† Volume of serum to volume of diluted serum.

‡ Mobilities, derived from descending patterns, are expressed as sq. cm. per second per volt  $\times 10^5$ .

$\alpha$ -globulin somewhat similar to the  $\beta$  anomaly observed in the patterns of human sera (3), and similar to the one occurring at the  $\alpha$ -globulin position in thyroidectomized rats (17). It has been suggested<sup>1</sup> that, since the cholesterol content of serum increases with thyroidectomy, this  $\alpha$  spike might be due to a cholesterol-protein complex; but it has since been found that the cholesterol in both normal and thyroidectomized rat serum migrates with

<sup>1</sup> By Dr. Warren Sperry of the Psychiatric Institute, New York.

the albumin fraction.<sup>2</sup> In human serum, however, the cholesterol is associated with the globulin fraction.<sup>3</sup>

Of all the sera studied, the patterns for the laboratory rat (Long-Evans strain) and the cotton-rat probably differed most widely. The cotton-rat,

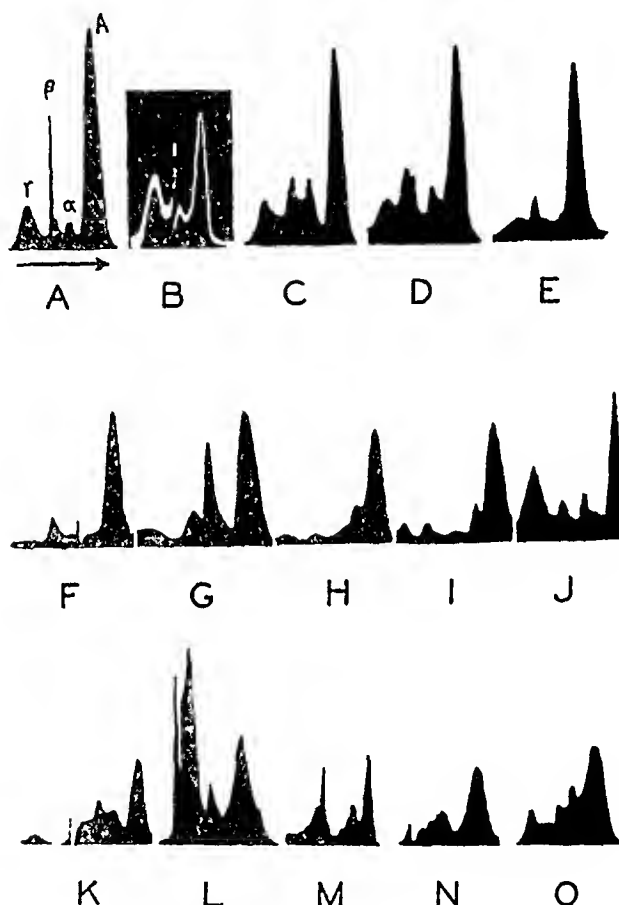


FIG. 1. Typical electrophoresis patterns of normal serum. *A*, man; *B*, monkey; *C*, swine; *D*, dog; *E*, rat; *F*, 21 day-old rat; *G*, cotton-rat; *H*, guinea pig; *I*, hamster; *J*, cat; *K*, 4 day-old kitten; *L*, chicken, hen; *M*, cockerel; *N*, female pigeon; *O*, carp. Buffer, 0.02 M sodium phosphate and 0.15 N NaCl; pH 7.4.

instead of having little or no  $\alpha$ -globulin, had a component in this region which constitutes one-fourth to one-third of the total pattern area.

The guinea pig and hamster sera (Syrian, golden) are characterized by

<sup>2</sup> Smelser, G. K., Sperry, W., and Moore, D. H., unpublished data.

<sup>3</sup> Sperry, W., personal communication.

two components in the  $\alpha$ -globulin region but the sera of the two species differed in that the  $\beta$ - and  $\gamma$ -globulins were almost missing in the guinea pig, whereas they were somewhat larger in the hamster, and Component 2 in the hamster serum was always much sharper than in the guinea pig. In order

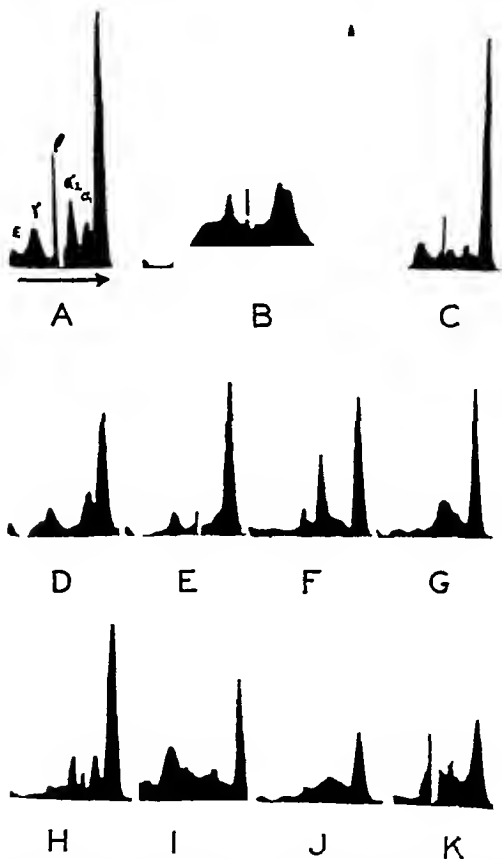


FIG. 2. Typical electrophoresis patterns of normal serum. *A*, man; *B*, swine; *C*, rabbit; *D*, rat; *E*, 21 day-old rat; *F*, cotton-rat; *G*, guinea pig; *H*, hamster; *I*, cat; *J*, 4 day-old kitten; *K*, carp. Buffer, 0.1 *N* sodium diethyl barbiturate and 0.02 *N* diethylbarbituric acid; pH 8.6.

to see whether Components 1 and 2 of hamster serum failed to be resolved at pH values nearer their isoelectric points, a sample was examined in the phosphate-NaCl buffer at pH 6.6 (Line 45, Table I). No change in the general character of the pattern was observed.



Cat sera also had an indication of two  $\alpha$ -globulins and an unusually large  $\gamma$ -globulin. The main component (albumin) of cat serum had the highest mobility of any sera studied. The serum of the 4 day-old kitten contained only traces of the two more slowly moving components, with Components 2 and 3 proportionally larger than in the adult cat (cf. Fig. 1, *J* and *K*).

It was found that there was a significant difference between the serum patterns of the male and female chicken, which was not found in any other species examined. Hen sera were obtained from fowls raised in the laboratory and also on a small and on a large chicken farm. Although there were slight variations in the serum patterns, all had the same general character. The patterns were characterized by two very large slow moving components and two faster moving components; of these last, the smaller was the faster. Both pairs of components were poorly resolved. A protein partition by the Howe method (23, 24) was made on the sample shown in Fig. 1, *L*<sub>4</sub> (Line 63, Table I). The quantities of albumin, pseudoglobulin, and euglobulin found were 1.7, 1.2, and 3.3 gm. per cent, respectively. Cockerel sera also had two faster moving components, the larger always being the faster. The patterns of both hen and cockerel sera contained spikes, indicating steep refractive gradients in the region of the slower moving components. One hen known to be ovulating (Line 63, Table I) was found to have a larger Component 5 than had non-ovulating hens.

The single sample of female pigeon serum examined was found to be very different from chicken serum.

Carp serum contained four components with comparatively large  $\alpha$ - and  $\gamma$ -globulins.

*Patterns in Barbiturate Buffer at pH 8.6*—In order to investigate the possibility of the appearance of new components and to obtain a more accurate measurement of the  $\gamma$ -globulin, several samples of serum from various species were examined in the barbiturate buffer at pH 8.6. It was observed by Longsworth (15) that human serum exhibited an extra component designated  $\alpha_1$ -globulin, which in other buffers with pH values less than 8.0 migrated with the albumin. Also in human serum the mobility of the  $\gamma$ -globulin was sufficiently increased to separate this component from the boundary caused by the buffer salts. It is of interest to note, however, that according to Lines 5 and 6 of Table I there is little difference in the quantity of each component measured in the two buffers except that Component 2 is separated from Component 1 in the buffer of higher pH and ionic strength. As is seen in Fig. 2, *B*, the sample of swine serum examined contained not only two  $\alpha$ -globulins but two or more  $\beta$ -globulins and an additional small component with mobility between that of albumin and  $\alpha$ -globulin. The two patterns (Figs. 1, *C* and 2, *B*; Lines 14 and 16, Table I) were obtained on the same sample of serum and illustrate the dependence of the electrophoretic pattern on the buffer.

Rabbit sera (Fig. 2, C) exhibited a Component 3 in this buffer with an indication of a Component 2, whereas in the phosphate buffer there was usually none. Rat sera (Fig. 2, D), however, had a large Component 2 with but an indication of Component 3. The  $\beta$ - and  $\gamma$ -globulins of rat serum, like those in swine serum, were not well resolved in this buffer.

Little difference from the patterns found in phosphate buffer was demonstrated in the serum of cotton-rats by the use of the barbiturate buffer of higher pH.

In guinea pig serum the ratio of the magnitude of Components 2 and 3 was consistently reversed by the use of the barbiturate buffer (*cf.* Figs. 1, H and 2, G).

Hamster serum (Fig. 2, H) exhibited well defined Components 2 and 4 in this buffer and the differences between hamster and guinea pig sera were more striking here than in the phosphate buffer.

The analyses of cat serum apparently can be made more satisfactorily in the phosphate buffer than in the barbiturate buffer. In the latter buffer the components were not resolved as well and no new ones appeared. In serum from a 4 day-old kitten the  $\gamma$ -globulin was found to be extremely low (Fig. 2, J).

The carp serum in barbiturate buffer (Fig. 2, K) resembled cockerel serum in the phosphate-NaCl buffer (Fig. 1, M).

#### DISCUSSION

From the foregoing data it is evident that many species under the conditions here studied present serum protein patterns which are so characteristic that it is possible to name the species and in some cases even the strain (rat) and sex (chicken) from a glance at the pattern.<sup>4</sup>

It is of interest to note the variation of  $\gamma$ -globulin with species and with age. From what is known of the quantity of antibodies in normal and immune animals, it is evident that the total quantity of  $\gamma$ -globulin cannot be used as a criterion of immunity. Indeed a considerable amount of evidence has accumulated to indicate that the quantity of  $\gamma$ -globulin is frequently altered (usually increased) under a variety of pathological conditions. For example, the amount of  $\gamma$ -globulin has been shown to increase consistently after hypophysectomy or thyroidectomy in rats (16,

<sup>4</sup> Many of the serum samples were taken from animals living on similar diets under similar conditions, and it is possible that sera from the same species living under different conditions might present a pattern different from the one here considered typical. It is also possible that the low albumin and high globulin of such animals as monkeys and cats may result from the animal being poorly adapted to laboratory cage life, whereas the rat, rabbit, and guinea pig may be well adapted. There is also evidence from the data on rats and cats, of which the young were studied, and from data on developing chick and pig embryos (18) that there are major changes in the serum patterns with age and development.

17) and also after hypophysectomy in monkeys.<sup>5</sup> Furthermore, it has been found that so called normal rats of the Sherman strain in this institution have from 3 to 5 times as much  $\gamma$ -globulin as do the rats of the Long-Evans strain.<sup>6</sup> The Sherman rats are known to be less hardy, slower growing, and significantly more susceptible to infection than the rats with the low globulin. Whether these differences are manifestations of strain characteristics or simply a result of dissimilar diet and care has not yet been determined.

Gutman *et al.* (25) have shown that in the sera of human subjects the quantity of albumin is consistently higher and the globulins consistently lower in the healthy state than that found in most pathological conditions. With but few exceptions this rise in globulin with disease is due at least in part to the  $\gamma$ -globulin fraction, as shown by Luetscher (5), Gray and Barron (26), Hanger *et al.* (27, 28), and others. This occurs in liver disease as well as in many chronic infections and in many disturbances in the reticulo-endothelial system, such as Hodgkin's disease and myeloma. Antibodies in sera usually migrate with a mobility near that of  $\gamma$ -globulin, as has been shown by Tischi and Kabat (2), Wyckoff *et al.* (6, 7), Cohn *et al.* (29), and others, but no electrophoretic differences can be detected between physiologically occurring  $\gamma$ -globulin and that containing immune bodies.

Many publications concerning the changes in human serum protein patterns have appeared (3, 5, 25-28, 30-33). In general, the electrophoretic method has not as yet proved more advantageous than the Howe fractionation method (23-25) in demonstrating serum protein changes occurring with any specific disease or clinical symptom; therefore great care must be taken in drawing conclusions from a small number of cases. As an example, multiple myeloma may produce in the serum patterns an abnormally high  $\beta$ -globulin or  $\gamma$ -globulin, new components (25, 32), or no deviation from the normal (31), the latter finding being the most common. Also in a recent patient similar drastic changes in the serum pattern were observed, even to the appearance of a major component between the  $\beta$ - and  $\gamma$ -globulin,<sup>7</sup> but at autopsy no myelomatoid tissue was demonstrated. Similar inconsistencies occur in other diseases. Patients with rheumatic fever (31), catarrhal jaundice (26), lymphatic leucemia,<sup>8</sup> Hodgkin's disease,<sup>8</sup> essential hypertension<sup>9</sup> may have sera with reduced albumin and elevation in all, any one, or none of the globulins, or no deviation from the normal at all. There seems to be as yet no definite relation of the serum protein pattern to any of these

<sup>5</sup> Smith, P. E., and Moore, D. H., unpublished data.

<sup>6</sup> The two strains of rats are under different care and on a different diet.

<sup>7</sup> Perera, G. A., and Moore, D. H., unpublished data.

<sup>8</sup> Kabat, E. A., personal communication of unpublished data.

<sup>9</sup> Bruger, M., and Moore, D. H., unpublished data.

diseases. The factor of the individual response to a stimulus seems to be of prime importance.

It may not be possible to determine the meaning of the variations of the various serum protein components with species and with various physiological or pathological states until the mechanism of serum protein production is revealed and the many factors involved in individual response to a stimulus are better understood. Until more is known, it is apparent that diagnosis of disease on the basis of electrophoretic patterns is fraught with many uncertainties.

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#### SUMMARY

Sera from each of a large number of animal species have been found to possess a characteristic and reproducible electrophoretic pattern. When examined under comparable conditions, the number and relative quantity of components varied significantly with the species. The sera of rats and guinea pigs contained little  $\gamma$ -globulin and that of baby rats and kittens contained the least  $\gamma$ -globulin of all. The variation of the serum protein components with physiological and pathological states is discussed.

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# EXPERIMENTALLY INDUCED CHANGES IN THE PROTHROMBIN LEVEL OF THE BLOOD

## I. QUANTITATIVE STUDIES IN DOGS GIVEN DICUMAROL

## II. THE EFFECT OF METHYLXANTHINES ON PROTHROMBIN PER SE AND WHEN ADMINISTERED WITH DICUMAROL\*

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### *I. Quantitative Studies in Dogs Given Dicumarol*

The great advances that have been made in recent years in the study of the coagulation reaction of the blood have come about largely from work done on prothrombin. In order that this progress continue, greater insistence on accuracy in the determination of prothrombin must be made and a more comprehensive view of the nature of prothrombin should be adopted. According to the evidence of the writer (1) prothrombin is a complex consisting of calcium and two components. Dicumarol, a drug which depresses prothrombin activity, does so by causing a reduction of one of these essential components, which has been designated as Component B. On the basis of this concept, it is possible to study more critically prothrombin activity following the administration of dicumarol. As a preliminary step certain basic factors such as the choice of animals, a correlation of the prothrombin level and the coagulability of the blood, and the effect of dosage were studied, and these results are presented in Part I of this paper.

*Selection of Animals*—The rabbit and the dog are the most suitable of the common laboratory animals for studying the effect of dicumarol. Both respond readily either to the oral or to the intravenous administration of the drug, as Link and his associates (2) have shown, blood is easily obtainable routinely by venipuncture, and rabbit brain thromboplastin is a specific activator of the prothrombin in both species. The chief disadvantage is that the prothrombin level of rabbit and dog blood is approximately 5 times that of human blood (3) and that, if this divergency is not fully considered when interpreting the results obtained in these animals in terms of human clinical problems, much confusion can result.

Limited studies suggest that the cat is even more susceptible to dicu-

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The dicumarol used in this study was kindly furnished by Eli Lilly and Company.

marol than the dog. The animal is unsuitable, however, because of the relative difficulty of getting blood. Heart puncture, it should be mentioned, invariably leads to fatal hemopericardium as soon as the prothrombin time is increased above 30 seconds, and therefore is not a safe procedure. In one experiment the prothrombin time was increased from the normal of 8 to 38 seconds in 48 hours by two successive daily oral doses of 5 mg. per kilo of body weight.

There are certain disadvantages in the use of the guinea pig and the rat for quantitative changes in the prothrombin level. It is relatively difficult to obtain blood except by heart puncture. The daily removal of 1 cc. of blood, the amount required for analytical checks, from a small animal may introduce a secondary factor; namely, the effect of excessive loss of blood. Rabbit brain thromboplastin is not a specific activator of guinea pig and rat prothrombin. Reliable results in these animals can be expected only if autogenous thromboplastin is employed.

The chicken appears to be particularly resistant to dicumarol. After feeding roosters 10 mg. per kilo of body weight daily for 6 days, no significant lowering of the prothrombin level occurred. After several daily intravenous injections of 10 mg. per kilo the maximum prothrombin time obtained was 15 seconds, which returned to the normal of 10 seconds in 24 hours after the last injection of the drug.

*Correlation of Prothrombin Time with Coagulation Time and Other Variables*—In order to determine the quantitative relationship of the prothrombin time to coagulability of the blood, the Lee-White and the clotting time of recalcified plasma were selected (4) as probably the most reliable and practical methods. In a few dogs the hematocrit reading and the sedimentation rate were noted, but as no significant changes were observed, extensive study was not deemed necessary. The hematocrit reading is useful primarily for detecting internal hemorrhage early and promptly.

From the results recorded in Table I, it will be observed that, while no exact relationship exists between the prothrombin time and the coagulation times of recalcified plasma and of whole blood, there is nevertheless a progressive increase in the latter two as the prothrombin of the blood drops. One should not expect a close correlation between the clotting time and the prothrombin level, since coagulation depends on other factors, particularly the release of thromboplastin. Prothrombin exerts its influence only after a marked reduction has occurred. The clotting time is little affected until the prothrombin time has been increased to about 20 seconds, which is equivalent to approximately 5 per cent of the normal level. While it is true that the author's method on undiluted plasma cannot detect changes until the prothrombin drops to 50 per cent, there is no good evidence that any decrease less than this has much practical significance. In fact even a

decrease of 80 per cent merely brings the level to that of human blood. When the prothrombin drops below 50 per cent the prothrombin time becomes sufficiently prolonged to allow accurate timing without resorting to dilution of the plasma. It is by far more expedient to master the tech-

TABLE I  
*Correlation of Progressive Fall in Prothrombin Level Due to Dicumarol with Clotting Time, Clotting Time of Recalcified Plasma, Hematocrit Reading, and Sedimentation Rate*

Day	Dog*	Prothrombin time† sec.	Clotting time (Lee-White) min	Clotting time of recalcified plasma sec	Hematocrit reading	Sedimenta- tion rate mm.
1	A	6 (100)	3.5	65		
	B	6 (100)	3.5	55		
	C	6 (100)	4	75	51	1
	D	6 (100)	4	60	48	1
	A	10.5(22)	3.5	72		
	B	12.5(14)	3.5	85		
2	C	13 (13)	4	50	52	1
	D	11 (20)	4	60	50	10
	A	16 (9)	4	140		
	B	19 (6)	4	105		
	C	23 (4.5)	4	70	42	7
	D	23 (4.5)	4	90	46	2
3	A	23 (4.5)	5	105		
	B	23 (4.5)	4.5	100		
	C	35 (2)	5	125	44	4
	D	31 (3)	5	150	50	5
	A	43 (1.5)	7.5	165		
	B	55 (1)	5.5	210		
4	C	75 (>1)	5.5	255	45	4
	D	60 (>1)	5.5	270	47	1
	A	47 (1)	8	205		
	B	100 (>1)	8	630		
	C	85 (>1)	12.5	270	33	5
	D	60 (>1)	7.5	210	45	2

\* The weights of Dogs A, B, C, and D were 5.25, 6.5, 11, and 11.5 kilos respectively.

† The figures in parentheses are the prothrombin concentration in per cent of normal.

nique of the original method than to depend on the dilution of plasma and thereby incur the risk of introducing an error resulting from the disturbance of the physicochemical relationship of the components of the plasma.

When the prothrombin is markedly reduced, the coagulation time becomes greatly delayed, as the following experiment illustrates. Dog 15, weight 7 kilos, was fed 10 mg. of dicumarol per kilo of body weight for 5



days. On the 7th day, the prothrombin time was 19 minutes, the coagulation time (Lee-White) 20 minutes, the clotting time of recalcified plasma 30 minutes.

After a transfusion of 35 cc. of normal dog blood mixed with 5 cc. of 0.1 M sodium citrate, the prothrombin time was 21 seconds, the coagulation time (Lee-White) 5.75 minutes, the clotting time of recalcified plasma 3 minutes.

Obviously when the prothrombin time increased to 19 minutes, the blood contained only a trace of prothrombin. On the assumption that the animal had a blood volume of 560 cc. (8 per cent of body weight) the addition of 35 cc. of normal blood containing 100 per cent prothrombin should, on the basis of calculation, have elevated the prothrombin to 5.8 per cent. Actually, a prothrombin time of 21 seconds was obtained which is equivalent approximately to 5 per cent. This is a surprisingly good agreement and attests to the quantitative reliability of the one-stage method.

*Effect of Dosage*—In 1937 the writer (5) observed that when toxic sweet clover was fed to rabbits, the prothrombin stayed normal for 24 hours, then dropped rather abruptly to about 20 per cent of normal in 48 hours, and after that continued to decrease at a slower but rather characteristic rate. After Link's work (6) made the active principle of toxic sweet clover hay, dicumarol, available, various investigators studied the quantitative effect of the drug on the prothrombin level (7). Link and his associates made the most extensive investigations but, instead of using the original one-stage method, they modified the procedure and expressed most of their results as the prothrombin time of 12.5 per cent plasma (1 part diluted to 8 with saline solution (8)). It is difficult to calculate this empirical figure in terms of prothrombin concentration and there is furthermore a possibility that such a high dilution may sometimes lead to errors.

Link and his coworkers (2) were the first to study the effect of a single dose of dicumarol in various animals. This procedure is particularly useful to standardize the action of dicumarol. In the present investigation the drug was administered intravenously for the most part, since Link and his group (2) have shown that when large doses are fed to dogs, an appreciable amount escapes absorption.

The first task was to find the minimum quantity of dicumarol that caused a demonstrable diminution of prothrombin. Link's group (2) stated that a 10 mg. dose in an 8 to 10 kilo dog produced a detectable effect. In this study it was likewise found that 1 mg. per kilo of body weight produced in some dogs a slight decrease in prothrombin even after one dose, and that if that dose was continued daily a distinct and progressive drop occurred in the majority of the animals, as shown in Table II. This suggests that the organism's capacity to destroy or eliminate the toxic agent is slightly

less than 1 mg. per kilo and that therefore if that dose is exceeded an accumulative effect results. It will be observed that certain dogs such as Dog B are very resistant, while others are fairly susceptible. Link (8) was the first to observe such refractoriness in rabbits. From the present results, it can be concluded that one cause for resistance is a highly developed detoxication mechanism in the organism. Since the response from the oral dose was essentially the same as from the intravenous, it can be concluded that small doses are completely absorbed from the intestinal tract.

TABLE II

*Effect of Administering Dicumarol to Dogs in Dosage of 1 Mg. per Kilo of Body Weight Daily*

Day	Prothrombin concentration									
	Dog K		Dog M		Dog P		Dog S		Dog B	
	Intra-venous*	Oral	Intra-venous	Oral	Intra-venous	Oral	Intra-venous	Oral	Intra-venous	Oral
1	100	100	100	100	100	100	100	100	100	100
	(6)†	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)	(6)
	100	100	50	100	100	80	100	100	100	100
2	(6)	(6)	(7)	(6)	(6)	(6.5)	(6)	(6)	(6)	(6)
	50	50	20	35	40	40	30	40	80	100
	(7)	(7)	(11)	(8.5)	(8)	(8)	(9)	(8)	(6.5)	(6)
3	20	50	10	25	15	30	13	30	50	100
	(11)	(7)	(15)	(9.5)	(12)	(9)	(13)	(9)	(7)	(6)
	15	30	9	10	13	20	13	10	40	100
4	(12)	(9)	(16)	(15)	(13)	(11)	(13)	(15)	(8)	(6)
	15	15	6	7	7	7.5	9	7	30	100
	(12)	(12)	(19)	(18.5)	(18)	(16.5)	(16)	(18.5)	(9)	(6)

\* The drug was given intravenously as the sodium salt.

† The figures in parentheses are the prothrombin times in seconds.

As the single dose is increased, the rate of drop in prothrombin is not altered but the period in which the drop occurs as well as the recovery time becomes longer (Fig. 1). It can be seen in Table I that certain dogs are more resistant, but in these the resistance is not brought about by a more rapid destruction of the drug, but is due to a decreased response to the dicumarol. The period of recovery, however, is no doubt dependent on the speed of detoxication. In the majority of dogs the prothrombin drops to about 20 to 25 per cent of normal in 24 hours and to 5 to 10 in 48 hours. After this time, even when a dose of 20 mg. per kilo is given, the recovery stage sets in and the prothrombin gradually returns to normal. If, instead of a single dose, dicumarol is given daily, the prothrombin continues

to drop as shown in Table I. In adult dogs the maximum reduction in 24 hours irrespective of the dose is about 80 per cent and in 48 hours 95 per cent. In puppies a greater reduction is often observed. This suggests that dicumarol merely stops the production of prothrombin and that the actual decrease represents the body's normal consumption or destruction of this clotting agent under ordinary physiological conditions.

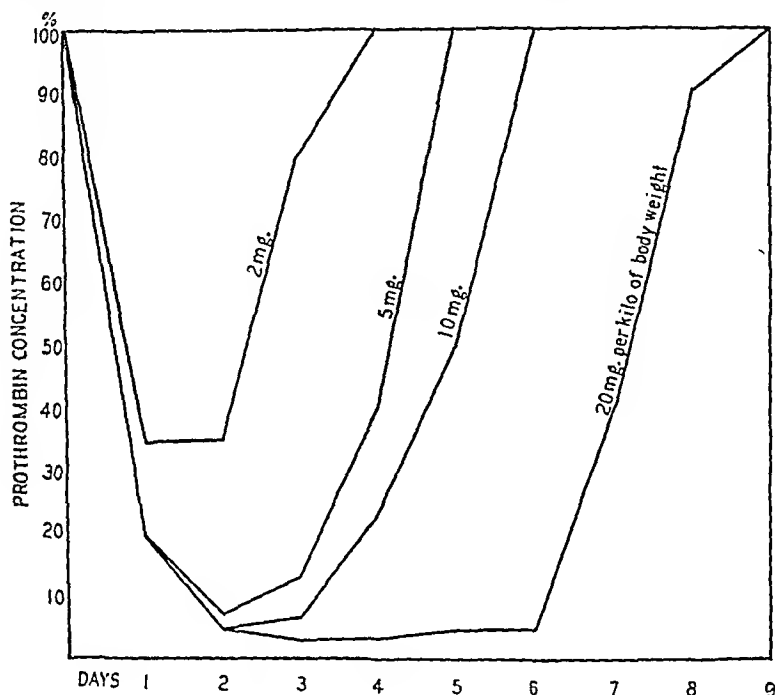


FIG. 1. The effect of increasing single doses of dicumarol on the prothrombin concentration. The drug was given intravenously. A small percentage of dogs is more resistant to dicumarol than the dog employed in this chart.

## II. The Effect of Methylxanthines on Prothrombin *per Se* and When Administered with Dicumarol

Field, Larsen, Spero, and Link (9) reported that the methylxanthines, caffeine, theobromine, theophylline, and preparations containing these compounds induced a state of hyperprothrombinemia when fed to dogs, rabbits, and rats, and furthermore counteracted the hypoprothrombinemic action of dicumarol. Since these findings are not only of theoretical interest but also of clinical importance, this problem was reinvestigated.

The experimental work was done only on dogs and rabbits for reasons

discussed in Part I. The prothrombin was determined by the writer's method on undiluted plasma and also on plasma diluted 1:5 with saline (20 per cent) and 1:20 with human plasma (5 per cent) treated with aluminum hydroxide. The latter medium is particularly satisfactory, since minute changes in the concentration of Component B can be detected. Plasma treated with aluminum hydroxide lacks Component B but still contains Component A. It moreover has a normal concentration of fibrinogen, thus maintaining very closely the physicochemical composition of undiluted plasma. The 5 per cent plasma normally clots in 18 to 21 seconds. The variation is due mainly to small differences in the potency of the thromboplastin.

From a study of Tables III and IV it can be clearly seen that the three methylxanthines did not cause any definite and demonstrable increase in the prothrombin level as measured by the original one-stage method on either undiluted or diluted plasma. These observations do not agree with those of Field, Larsen, Spero, and Link. While the reason for these differences is not clear, it is probable that they can be accounted for by the modifications which were introduced by Link and his associates (8) in the writer's method for the determination of prothrombin. They use a thromboplastin much less active than that recommended and consistently used by the author. The normal prothrombin time for undiluted plasma as recorded in Fig. 1 of the paper of Field *et al.* (9) is 12 to 13 seconds, whereas with the writer's thromboplastin it is 6 seconds. It should further be noted that the normal times for 12.5 per cent of dog plasma which they record (9, 11) range from 20 to 35 seconds, which is a variation of 75 per cent. In the original one-stage method such fluctuations in dog blood are not observed. The clotting time usually is 6 and only occasionally 6.5 seconds. On the basis of a 75 per cent variation in range, the prothrombin time would be 6 to 10.5 seconds, but actually the latter clotting time is obtained only when the prothrombin level has decreased to 22 per cent of normal. In rabbits the prothrombin time of undiluted plasma likewise is 6 seconds, but occasionally it may be increased to 7 or 7.5 seconds for no explainable reason. It is not due to a lack of vitamin K.

Since the prothrombin time of normal 12.5 per cent dog plasma has a wide range, great caution is necessary in drawing conclusions from results such as recorded by Field and his associates. An analysis of their data reveals that in only one instance did a methylxanthine reduce the prothrombin time lower than that of one of their normal controls, which was 20 seconds (11). Even after giving 400 mg. of theophylline per kilo of body weight the prothrombin time of 12.5 per cent plasma only dropped from 31 to 22.6 seconds, whereas the normal prothrombin time of three of their dogs ranged from 20 to 22.8 seconds. Such results do not appear

striking enough to justify their statement that "it is conceivable that their use in man might augment the tendency for thrombus formation." Partic-

TABLE III

*Effect of Caffeine, Theobromine, and Theophylline on Prothrombin Level of Dogs*

Dog	Prothrombin time				Remarks
	Dilution of plasma				
	100	50*	20*	5†	
	sec.	sec.	sec.	sec.	
M	6	7	11	21	Control
"	6	7	11	18	200 mg. caffeine per kilo body weight
S	6	7	11	20	Control
"	6	7	11	20	200 mg. theobromine per kilo body weight
F	6	7	11	20	Control
"	6	7	11	19	200 mg. theophylline per kilo body weight
After 24 hrs.					
M	6	7	11	18	Control
"	6	7	10.5	19.5	Caffeine
S	6	7	11	19.5	Control
"	6	7	11	18	Theobromine
F	6	7	12	19	Control
"	6	7	10	19	Theophylline
After 48 hrs.					
M	6	7	9.5	20	Control
"	6	7	10.5	19	Caffeine
S	6	7	12	19	Control
"	6	7	11	19	Theobromine
F	6	7	12	18.5	Control
"	6	7	12.5	22	Theophylline
After 72 hrs.					
M	6	7	12	20	Control
"	6	7	11	20	Caffeine
S	6	7	11.5	20	Control
"	6	7	11	19	Theobromine
F	6	7	13	21	Control
"	6	7	11	19	Theophylline

\* Diluted with 0.85 per cent sodium chloride solution.

† Diluted with human plasma treated with aluminum hydroxide according to the writer's method (10).

ularly unwarranted is their statement "that the methylxanthines not only render the blood hypercoagulable." There are no experiments recorded in their paper that indicate that the clotting time of blood was

studied. This is still the only recognized means for measuring coagulability. Hyperprothrombinemia is not synonymous with hypercoagulability; in fact, the prothrombin can fluctuate within a wide range without affecting the clotting time, as shown in Table I. It is not the prothrombin level, unless markedly reduced, which determines the clotting time of the blood. The most important factor is the speed with which thromboplastin

TABLE IV

*Effect of Caffeine, Theobromine, and Theophylline on Prothrombin Level of Rabbits*

Rabbit No.	Prothrombin time				Remarks
	Dilution of plasma				
	100	50*	20*	5†	
	sec.	sec.	sec.	sec.	
1	6.5	7	10.5	20	100 mg. caffeine per kilo body weight
2	6	7	9	20	200 " theobromine per kilo body weight
3	6.5	7	10.5	19	200 " theophylline " " " "
4	6	7	10	18	Control
5	6.5	7	11	21	"

After 24 hrs.

1	6.5	7	10.5	20.5	Caffeine
2	6	7	10	20	Theobromine
3	6	7	11	17.5	Theophylline
4	6	7	10.5	20.5	Control
5	6.5	7	11	21.5	"

After 48 hrs.

1	6.5	7	9.5	20	Caffeine
2	6	7	10.5	18.5	Theobromine
3	6	7	11	19	Theophylline
4	6	7	9.5	20	Control
5	6.5	7	10	20	"

\* Diluted with 0.85 per cent sodium chloride solution.

† Diluted with human plasma treated with aluminum hydroxide.

is released that governs the coagulation time according to the most widely accepted concept of the clotting mechanism.

It is not the purpose of this discussion to enter into a polemic, but merely to consider the methylxanthines from the point of view of safety in therapy. These drugs occupy a place in modern therapeutics and are widely used. If there is cause to believe that they tend to induce intravascular clotting, their employment should immediately be limited. From the standpoint of recorded clinical observations, there is no clear cut evidence that incriminates these drugs, and the experimental data

concerning their influence on the prothrombin level cannot be considered adequate to regard them dangerous as a causative factor in thrombosis.

TABLE V

*Influence of Methylxanthines in Counteracting Hypoprothrombinemic Action of Dicumarol\**

Dog	Prothrombin concentration, † per cent of normal						
	0 hr.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	
F	100 (6)	35 (8.5)	30 (9)	50 (7)	100 (6)		Control
M	100 (6)	30 (9)	13 (13)	8 (17)	20 (11)	30 (9) ‡	200 mg. theobromine per kilo body weight
W	100 (6)	15 (12)	10 (15)	40 (8)	100 (6)		Control
L	100 (6)	15 (12)	12 (13.5)	50 (7)	100 (6)		200 mg. theophylline per kilo body weight
F§	100 (6)	20 (11)	25 (9.5)	30 (9)	80 (6.5)	100 (6)	200 mg. caffeine per kilo body weight
M	100 (6)	22 (10.5)	9.5 (15.5)	7 (18.5)	11.5 (14)	30 (9)	Control
W	100 (6)	13 (13)	4 (24)	4 (24)	40 (8)	100 (6)	200 mg. theobromine per kilo body weight
L	100 (6)	15 (12)	4 (25)	7 (18)	40 (8)	100 (6)	Control
F	100 (6)	30 (9)	25 (9.5)	40 (8)	100 (6)		"
M	100 (6)	20 (11)	8 (17)	8.5 (16.5)	23 (10)	30 (9)	200 mg. theophylline per kilo body weight
W	100 (6)	14 (12.5)	5.5 (20)	30 (9)	100 (6)		Control
F	100 (6)	30 (9)	20 (11)	30 (9)	80 (6.5)	100 (6)	20 mg. theobromine per kilo body weight
M	100 (6)	23 (10)	7.5 (17.5)	6 (19)	15 (12)	23 (10)	Control
F	100 (6)	30 (9)	30 (9)	50 (7)	100 (6)		"
M	100 (6)	20 (11)	7.5 (17.5)	5 (21)	22 (10.5)	30 (9)	100 mg. caffeine per kilo body weight

\* Each dog was given 5 mg. of dicumarol per kilo of body weight intravenously.

† The figures in parentheses are the prothrombin time in seconds.

‡ The prothrombin concentration usually became normal on the 7th day.

§ The animals were allowed to rest 1 week after the prothrombin level was restored.

This does not imply that these drugs are given *carte blanche*. They are exceedingly active and can produce toxic symptoms. Several animals,

including two young healthy dogs, were killed by caffeine in this study, and theophylline occasionally caused vomiting.

*Effect of Methylxanthines on Hypoprothrombinemia Induced by Dicumarol*—In order to test whether caffeine, theobromine, and theophylline prevented or counteracted the hypoprothrombinemia caused by dicumarol, a series of dogs was standardized by giving them 5 mg. of dicumarol, as the sodium salt, per kilo of body weight intravenously. The prothrombin was determined daily until the normal level was restored. The methylxanthines were fed immediately before or after the injection of dicumarol. The dose was 200 mg. of the free alkaloid per kilo of body weight, except for caffeine, which because of its toxicity was given in a dosage of 100 mg. during the latter part of the study.

No evidence whatsoever was found indicating that caffeine, theobromine, or theophylline counteracted dicumarol. In Table V a typical series of experiments is presented. The dogs used were representative of the variations observed in the study of this species during the past 3 years. Dog F was very resistant to dicumarol, while Dog M on the contrary was affected somewhat more than the average dog. Dogs B and L were brother and sister. They had the same weight and were very similar except for sex. Their response to dicumarol was almost identical. Neither theobromine nor theophylline had any inhibitory effect on dicumarol, as shown by using one as a control and feeding the other the methylxanthine. Unfortunately further study on these dogs was stopped since both were killed by caffeine, one by a dose of 300 mg. per kilo of body weight given in divided doses in 36 hours and the other by 200 mg.

All determinations of prothrombin were done by the author's original method on undiluted plasma within an hour after the blood was collected. Nearly every result was checked by repeating the estimation on plasma diluted with an equal volume of saline solution. Uniformly consistent results were obtained with the diluted and undiluted plasma. Occasionally the accuracy of the determination was further checked by mixing known volumes of plasmas containing different concentrations of prothrombin and then estimating the prothrombin of the mixture as illustrated by the following example.

Plasma 1	(prothrombin time 31 sec. = 3 % prothrombin)	1.8 cc.
" 2	" " 9 " = 30 %	" 0.2 "
Mixture of Plasma 1 + 2	" " 21 " = 5.0%	" "
By calculation	" " 20 " = 5.7%	" "

The failure to secure any evidence that the methylxanthines protect the dog against the hypoprothrombinemic effect of dicumarol is in complete



disagreement with the findings of Field, Larsen, Spero, and Link. It is difficult to account for this since their experimental conditions were essentially the same as the writer's, except that they fed the dicumarol instead of injecting it intravenously. Since administration by the latter route eliminates any uncertainty of absorption it seemed the preferred method, but to rule out the possibility that the difference in results was due to the mode of administration, a small series was run in which dicumarol was given orally. The results were the same as those recorded for the intravenous experiments.

#### SUMMARY

1. The dog and rabbit are suitable animals for the study of the action of dicumarol, as they respond promptly and consistently. The chicken is markedly resistant to the drug.

2. When the prothrombin time becomes prolonged, as the result of dicumarol, the coagulation time (Lee-White) and the clotting time of recalcified plasma also become progressively delayed.

3. The minimum dose of dicumarol which has a demonstrable effect on the prothrombin level of dogs is approximately 1 mg. per kilo of body weight. The maximum reduction of prothrombin is approximately 80 per cent in 24 hours and 95 per cent in 48 hours. This rate of prothrombin decrease cannot be accelerated by increasing the dose of dicumarol.

4. No evidence was obtained that caffeine, theobromine, and theophylline, when fed in large doses (100 to 200 mg. per kilo of body weight), increased the prothrombin level of the blood in dogs and rabbits.

5. Caffeine, theobromine, and theophylline had no protective action against the hypoprothrombinemic effect of dicumarol as measured on standardized dogs with a fixed and constant dose of the drug.

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# THE RELATIONSHIP BETWEEN VITAMIN M AND THE LACTOBACILLUS CASEI FACTOR\*

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Evidence of a more or less indirect nature has accumulated which tends to show a relationship between vitamin M (1) and certain of the factors which stimulate bacterial growth. Thus, the Ohio group (2) treated cytopenic monkeys with a "folic acid" concentrate prepared from yeast by the method of Hutchings *et al.* (3) and obtained a remission of the blood dyscrasia. Waisman and Elvehjem (4) obtained what they considered a significant weight response in monkeys by the use of a norit eluate of solubilized liver. It had previously been shown that a commercially available crude liver extract was capable of producing a prompt remission in the cytopenic monkey (5). In none of these experiments was a highly purified material used for the tests, however.

Evidence of another nature also pointed to the identity of vitamin M with the factor stimulating *Streptococcus faecalis*. Although the occurrence of vitamin M in certain crude materials could not be correlated with the *Streptococcus faecalis* factor content of these materials (6), it was found that after incubation with an enzyme prepared from rat liver (7) there was good agreement between the monkey assay and the microbiological assay (8).

It is obvious that the crucial test of the identity of vitamin M with any of the factors stimulating bacterial growth would be experiments wherein a pure substance, known to be microbiologically active, is shown to be biologically active in the monkey. It is the purpose of this paper to report such experiments, and to interpret them in the light of some of our microbiological studies.

## EXPERIMENTAL

Through the courtesy of Dr. E. L. R. Stokstad of the Lederle Laboratories, Inc., a quantity of *Lactobacillus casei* factor (9) was made available for experiments upon the monkey. Six curative type experiments have been made upon four animals. The most significant data from two of these experiments were reported previously (10). Complete hematological data

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on five of the experiments are plotted in Fig. 1 to 3 of this report. Weight records were kept but are not reported here. The day to day variation is so great that we do not consider weight changes in cytopenic monkeys to be as significant as blood picture changes, especially in short term curative type experiments.

The methods of housing and the care of the monkeys were the same as those previously used (1, 5, 6). In the hematological studies the methods outlined by Shukers *et al.* (11) were employed. The animals received Diet 600 with pure vitamin supplements (6).

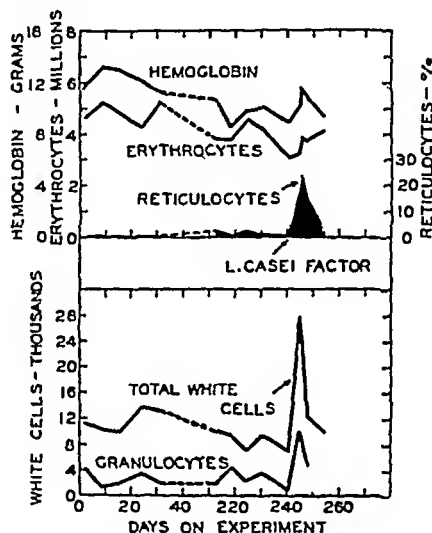


FIG. 1. Data on Monkey 146, which was given 1 mg. of *Lactobacillus casei* factor on the 242nd day and 2 mg. on the 243rd day, intramuscularly (total dose, 3 mg.).

The *Lactobacillus casei* factor was given by intramuscular injection in the doses indicated in the legends to Figs. 1 to 3. Marked increases in the numbers of all types of blood cells followed the injections. The six experiments were performed on monkeys with varying degrees of anemia at the time treatment began; initial erythrocyte counts ranged from 4.0 million per c.mm. in the first test on Monkey 147 (Fig. 3) to 1.5 million per c.mm. in the second test on Monkey 131 (Fig. 2). As would be expected, the reticulocyte responses bore an inverse relation to the initial erythrocyte counts; in the first test on Monkey 147 the maximum reticulocytes were 7.8 per cent; in the second test on Monkey 131 the reticulocytes reached a maximum of 47 per cent of the circulating erythrocytes. All of the monkeys showed a distinct leucopenia when used for the *Lactobacillus casei* factor test. Treatment was followed by sharp increases in the number of circulating white blood cells in every experiment.

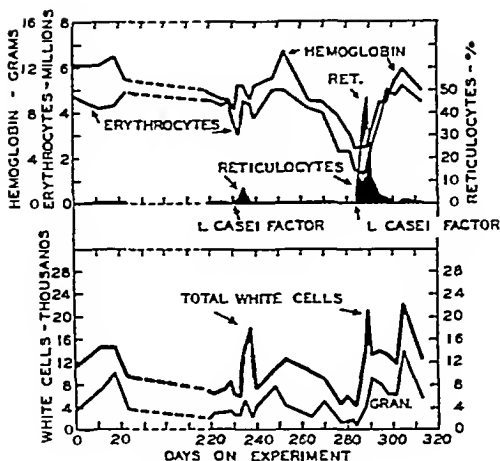


FIG. 2. Two experiments on Monkey 131. In the first experiment it received 2 mg of *Loctobocillus casei* factor on the 231st day and 1 mg on the 233rd day (total dose, 3 mg). In the second experiment it received 1.5 mg of *Lactobocillus casei* factor on the 235th day, 1 mg on the 289th day, and 0.7 mg on the 291st day (total dose, 3.2 mg).

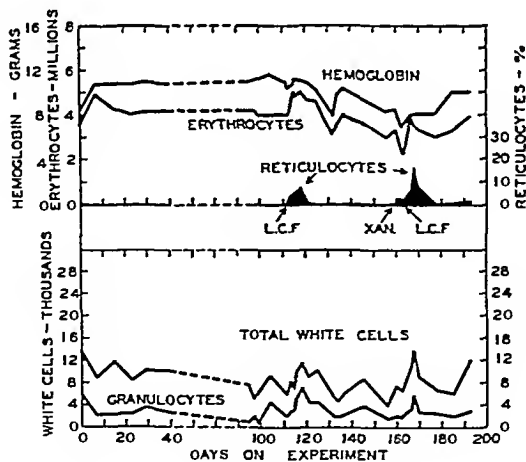


FIG. 3. Two experiments with *Lactobacillus casei* factor and one with xanthopterin on Monkey 147. In the first experiment it received 1 mg of *Lactobacillus casei* factor on each of the following days: 111th, 112th, and 113th (total dose, 3 mg). It received xanthopterin by injection as follows: 158th day, 1 mg; 160th day, 2 mg; 161st day, 3 mg; 162nd day, 4 mg; 163rd day, 3 mg (total dose, 13 mg). It received 1 mg of *Lactobacillus casei* factor on the 164th day and 1.8 mg on the 165th day (total dose, 2.8 mg).

The treatment of the cytopenic monkeys with *Lactobacillus casei* factor was followed by dramatic improvement in the clinical condition of the animals as well as in the blood picture. Several of the animals had exhibited loss of appetite, mild necrosis of the gums, and severe diarrhea when they were used for test. Treatment was followed by the prompt return of normal appetite, disappearance of diarrhea, and regression of the gum changes.

From the data given in the legends to Figs. 1 to 3, it will be seen that the total dose of *Lactobacillus casei* factor varied from 2.8 to 3.2 mg. Such doses resulted in maximum reticulocyte responses on the 4th day in five experiments, and on the 7th day in one experiment.

Monkey 147 (Fig. 3) received a total of 13 mg. of synthetic xanthopterin by intramuscular injection between the 158th and the 163rd days. Although there appeared to be a slight reticulocyte increase during this period, there was no evident effect upon the erythrocyte count, the white cell counts, or the hemoglobin level. This and another similar experiment would seem to indicate that *injected* xanthopterin is not as effective as xanthopterin given by mouth, as reported by Totter *et al.* (6). This difference may be due to the fact that, because of its relative insolubility, the dose of xanthopterin which can be given intramuscularly is necessarily much smaller than that which can be given by mouth.

Monkey 131 exhibited evidence of vitamin K deficiency on the 288th day, a massive subcutaneous hemorrhage and prolonged bleeding at the site where the ear vein was incised to draw blood for hematological studies. 2.5 mg. of menadiolone were injected on this day and again on the 289th. This treatment was followed promptly by correction of the bleeding tendency. Unfortunately, prothrombin time determinations were not made on the animal. In the 14 years of experience of this laboratory with vitamin M-deficient monkeys, this was the first case of prolonged clotting to be seen. We have since observed it in another monkey under somewhat similar dietary conditions.

The *Lactobacillus casei* factor used in this study was reported by Hutelings *et al.* to be relatively inert in stimulating the growth of *Streptococcus faecalis* (9); the amount required for half maximum stimulation of *L. casei* was 0.000061  $\gamma$  per ml., while the amount for half maximum stimulation of *S. faecalis* (*S. lactis*) was 0.0042  $\gamma$  per ml. We have likewise found that the *L. casei* factor has little stimulating effect for *S. faecalis*; under the conditions of our assay 0.0021  $\gamma$  per ml. is required for half maximum stimulation. However, after treatment with the rat liver enzyme of Mims *et al.* (7) the *L. casei* factor gave half maximum stimulation of *S. faecalis* in a concentration of only about 0.00009  $\gamma$  per ml.; this latter value is almost identical with the amount of a standard vitamin

B<sub>2</sub> sample required for half maximum stimulation of *S. faecalis* under similar conditions. The treatment with the rat liver enzyme thus effected a 23-fold increase in the ability of the substance to stimulate the growth of *S. faecalis*.

#### DISCUSSION

The data presented in the foregoing figures and paragraphs furnish cogent evidence for the effectiveness of highly purified *Lactobacillus casei* factor in the treatment of the monkey made anemic and leucopenic by a vitamin M-deficient diet. Every criterion applied, response of reticu-

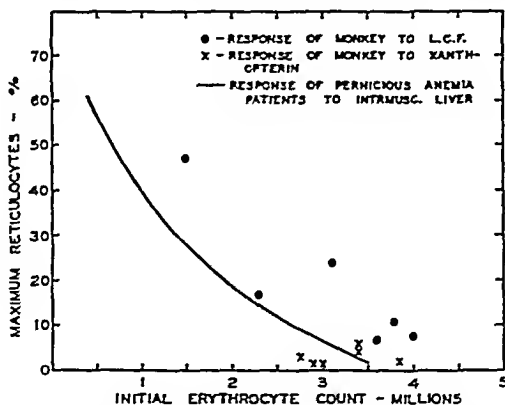


FIG. 4. Maximum reticulocyte responses (in per cent of total erythrocytes) plotted against initial erythrocyte counts (in millions per c.mm.). The filled circles represent responses of cytopenic monkeys to injected *Lactobacillus casei* factor, from data given in this paper. X represents the response to the feeding of xanthopterin to cytopenic monkeys as reported by Totter *et al.* (6). The solid line is plotted from the data of Isaacs and Friedman (12) for the maximum reticulocyte percentage after intramuscular liver therapy in pernicious anemia.

locytes, erythrocytes, hemoglobin, total white blood cells and granulocytes, and improvement in clinical condition, supports this conclusion. It is evident that a total intramuscular dose of about 3 mg. of *Lactobacillus casei* factor, divided over several days, is adequate to produce a dramatic response in the cytopenic monkey and to keep the cytopenia in remission for a period of from 10 to 30 days.

The response shown by the reticulocyte level is probably the most sensitive criterion of effective treatment. We have plotted in Fig. 4 the maximum reticulocyte responses against the initial erythrocyte counts; *i.e.*, the erythrocyte count on the day that treatment was started in each case.

Since we have no guide to the prediction of a maximum possible reticulocyte response in the cytopenic monkey, we felt that it would be of interest to compare our data with the predictable maximum reticulocyte response in a blood dyscrasia in another primate. Consequently, we have plotted also in Fig. 4 the response of pernicious anemia patients to adequate intramuscular liver therapy, taken from the data of Isaacs and Friedman (12). This is of course not to be construed as a suggestion of the identity of pernicious anemia in man with nutritional cytopenia in the monkey. Even a cursory glance at Fig. 4 reveals that the responses of the monkeys to *Lactobacillus casei* factor were much greater than the responses of pernicious anemia patients to adequate therapy. In Fig. 4 are also plotted the reticulocyte responses of cytopenic monkeys to the feeding of xanthopterin, as published by Totter *et al.* (6). Although undoubtedly significant, they are smaller than those produced by *L. casei* factor.

The data reported in this paper add two important facts to the knowledge of the significance of the *Lactobacillus casei* factor (9). It was reported by Hutchings *et al.* (9) that the substance was active in stimulating growth of *L. casei* but relatively inert toward *Streptococcus faecalis* (*S. lactis* R). We have now shown that *after enzymic treatment* the substance is about as active as a vitamin B<sub>12</sub> standard in stimulating *S. faecalis*. It was reported to be effective in the anemic chick (9), and was found by Daft and Sebrell to be active in the succinylsulfathiazole-treated rat (13). We have now shown it to be effective in the cytopenic monkey. Under appropriate conditions, therefore, the substance is biologically active for *L. casei*, *S. faecalis*, the anemic chick, the succinylsulfathiazole-treated rat, and the monkey.

The relation of vitamin M to other substances of microbiological importance is also of interest. The final evidence for the activity of vitamin B<sub>12</sub> and vitamin B<sub>12</sub> conjugate in the cytopenic monkey must of course await the actual use of the pure compounds in monkey experiments. Certain inferences can be drawn from known facts, however. Totter *et al.* (6) found that the content of substances stimulating *Streptococcus faecalis* in yeast was not sufficient to account for its effectiveness in preventing nutritional cytopenia in the monkey. However, if an extract of the yeast was first treated with an enzyme prepared from rat liver (7) the *S. faecalis*-stimulating activity of the yeast then measured was consistent with its vitamin M content (8). Similarly, the content of *S. faecalis*-stimulating factor in Difco yeast extract before enzymic treatment (preformed factor content) was not sufficient to explain the activity of yeast extract in the succinylsulfathiazole-treated rat, whereas the content of *S. faecalis*-stimulating factor after enzymic treatment (potential factor content) was sufficient to explain such activity (8). Thus yeast (and to a less extent

many other materials) contains a substance which is microbiologically inactive, but which is active in the cytopenic monkey and in the succinyl-sulfathiazole-treated rat; this substance becomes microbiologically active after enzymic treatment. It would appear to be a material with microbiological properties similar to those of vitamin B<sub>12</sub> conjugate (14). In this connection it should be recalled that the enzyme from rat liver which in this paper is reported to increase the activity of *Lactobacillus casei* factor in stimulating the growth of *S. faecalis* was previously shown to have a similar action on vitamin B<sub>12</sub> conjugate (15).

For the nutrition of higher forms of life (chick, rat, and monkey), it would appear that *Lactobacillus casei* factor, vitamin B<sub>12</sub>, and vitamin B<sub>12</sub> conjugate are interchangeable.

#### SUMMARY

With highly purified *Lactobacillus casei* factor, six curative type experiments have been performed upon four young rhesus monkeys, previously rendered anemic and leucopenic by a vitamin M-deficient diet. The *L. casei* factor was given by intramuscular injection in a total dose in each experiment of about 3 mg., divided over several days. The treatment was followed in every case by prompt remission of the blood dyscrasia and dramatic improvement in the clinical condition of the animal. Reticulocytes increased to maximum levels of from 7 to 47 per cent of the circulating erythrocytes (normal, 0.2 to 0.3 per cent); the crises were reached in from 4 to 7 days. Erythrocyte counts, hemoglobin levels, and white blood cells increased to normal or near normal levels. The dose used was adequate to keep the monkeys in remission for periods ranging from 10 to 30 days. These experiments furnish convincing evidence of the effectiveness of highly purified *L. casei* factor in vitamin M-deficient monkeys.

The *Lactobacillus casei* factor is relatively inert in stimulating the growth of *Streptococcus faecalis*. However, after treatment with an enzyme prepared from rat liver, it becomes as active as a standard vitamin B<sub>12</sub> preparation in stimulating *S. faecalis*. This rat liver enzyme had previously been shown to liberate the *S. faecalis*-stimulating factor from vitamin B<sub>12</sub> conjugate and from yeast.

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# THE EFFECTS OF VARIOUS INHIBITORS OF CARBOHYDRATE METABOLISM, IN VIVO

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Much information concerning the pathways of carbohydrate metabolism has been obtained by the use of enzymic inhibitors, *in vitro*. However, with the exception of phlorhizin, few data are available describing the effects of these inhibitors, *in vivo*. Turner and Hulpieu (1) found that sodium cyanide administered to rabbits produced high blood glucose and lactic acid concentrations within 3 to 4 hours. Neuss (2) and Irving (3) reported high blood sugar values in rabbits given iodoacetate and noted that this could be prevented by insulin. Nishida (4) confirmed these findings and also described a marked drop in the alkaline reserve after iodoacetate administration, while Milhorat and coworkers (5) found that iodoacetate produced a creatinuria in dogs which was accompanied by a considerable increase in phosphate excretion. Stare and Baumann (6) reported hyperglycemia in rabbits dosed with sodium malonate and that this compound apparently counteracted the effects of insulin. Kaplan and Greenberg (7) observed that while sublethal doses of sodium fluoride caused a hyperglycemia in rats, lethal doses actually resulted in a terminal hypoglycemia. These authors also noted an increase in liver glucose-6-phosphate in fluoride-poisoned rats accompanied by a diminished concentration of liver inorganic phosphate. While malonate, fluoride, and phlorhizin inhibited adenosine triphosphate formation, iodoacetate did not appear to interfere with this process.

In the present work, the effects of fluoride, cyanide, azide, malonate, and iodoacetate on blood glucose, lactic acid, inorganic and total acid-soluble phosphate, and pyruvic acid were investigated.

## EXPERIMENTAL

Adult rabbits were used throughout this work. Because of the considerable variability of blood glucose and lactic acid values in this species, each animal was given 10 mg. of nembutal intravenously and 20 mg. subcutaneously about 1 hour before administration of the inhibitor to be studied. In a series of control rabbits this dosage was found sufficient to stabilize blood glucose at values below 130 mg. per cent and lactic acid at values below 30 mg. per cent without influencing the other blood com-

ponents. The dose of inhibitor varied with each substance. In each case the dose finally chosen was that which would prove fatal within 3 to 5 hours. These were, in mg. per kilo of body weight, NaF 250,  $\text{NaN}_3$  10, NaCN 1.2, sodium iodoacetate 80, sodium malonate 1500. In each case the drug was given in a final volume of about 50 cc. subcutaneously; the cyanide and azide were dissolved in 0.85 per cent NaCl, the others in distilled water. Blood samples were collected every 30 minutes, with heparin as the anticoagulant. When possible, the terminal sample was collected a few minutes before death by heart puncture. On other occasions the final sample was obtained from the heart or jugular vein within a few minutes after death. Blood samples taken in this manner from control animals which had been sacrificed by a blow on the head or a large overdose of nembutal did not vary appreciably from the normal values which had been established for these animals.

Blood glucose was estimated by a modification of the Folin micromethod (8), phosphate according to Fiske and Subbarow (9), lactic acid by the Elgart and Harris modification of the Mendel-Goldscheider procedure (10) and checked by the technique of Barker and Summerson (11), and pyruvate by the method of Friedemann and Haugen (12). No quantitative determinations of acetone bodies were run but spot tests for acetoacetic acid by a modification of the Rothera test (13) were routinely performed.  $\text{CO}_2$ -combining power was estimated with the Van Slyke apparatus.

*Sodium Cyanide*—Results with this drug were rather variable in that it was difficult to establish a single dose which would be fatal in somewhat more than 2 hours after administration. The most effective results were obtained by periodic dosage with sublethal quantities. A typical protocol obtained in this manner is shown in Table I. Blood glucose, lactate, and inorganic phosphate accumulated steadily throughout the experiment. Only a small increment in pyruvate or organic phosphate was noted. Spot tests for acetone bodies were consistently negative. For the last 40 minutes of the experimental period the animals' respiration was markedly increased; then the breath came in deep gasps for about 10 minutes before death. Essentially similar data were obtained from a rabbit which had fasted for 72 hours before administration of the cyanide. However, in this animal, there occurred an initial rise in blood glucose, reaching a maximum value of 150 mg. per cent in 2 hours, followed by a steady decrease so that the final value was 47 mg. per cent at death. The other data were quite similar to those shown in Table I.

*Sodium Azide*—The response to this drug was quite similar to that described for cyanide and a typical protocol for a well fed rabbit is shown in Table II. The initial drop in serum inorganic phosphate, followed by a rise to values decidedly above normal, was seen in all the animals studied.

When fasting animals were used the response was again like that found with cyanide. For example, one fasted animal that had been given 12 mg. of sodium azide per kilo showed the following hourly blood sugar values: 103, 268, 325, 248, 105 mg. per cent, and, at death 30 minutes later, 86. The rise in blood glucose commenced very rapidly after administration of the drug. Thus, in two animals which had been given 30 mg. per kilo and died 22 and 34 minutes later, blood glucose levels of 241 and 276 mg. per cent respectively were found.

TABLE I  
*Metabolic Effects of Sodium Cyanide*

A male rabbit, weight 2400 gm, was given 32 mg of NaCN subcutaneously every hour during the experiment

Time	Blood glucose	Serum inorganic P	Serum organic P	Blood lactic acid	Blood pyruvic acid
min	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
0	119	4.8	0.8	34	0.9
70	128	5.1	0.7	42	1.4
130	172	5.3	1.1	55	1.3
195	153	5.5	1.6	72	1.7
300	221	6.9	1.3	138	2.5
340	273	11.8	1.9	276	3.4

TABLE II  
*Metabolic Effects of Sodium Azide*

An adult female rabbit weighing 2830 gm was given 35 mg. of NaN<sub>3</sub> dissolved in 25 cc of 0.85 per cent NaCl

Time	Blood glucose	Serum inorganic P	Serum organic P	Blood lactic acid	Blood pyruvic acid
min	mg per cent	mg per cent	mg per cent	mg per cent	mg per cent
0	115	5.1	0.95	45	1.1
60	229	4.2	0.82	75	1.3
135	334	4.3	0.56	98	1.7
250	425	5.7	0.78	194	2.8
325	515	11.8	1.1	310	3.9

The effect of insulin on azide poisoning was studied in several rabbits. The results of one such experiment are shown in Table III. After obtaining a control blood sample, 30 units of insulin were given and the azide administered 60 minutes later. The lowering of serum inorganic phosphate usually seen after insulin administration was accentuated by the azide, and the terminal rise in phosphate was not as marked as usual. The blood sugar seemed to represent a balance between the actions of the two drugs,

while the lactic acid rise was unaffected by the insulin. Insulin was also given to several rabbits in which the azide-induced hyperglycemia was also established, with results much like those above. Thus in one rabbit which showed control glucose, phosphate, and lactate concentrations of 123, 4.35, and 36 mg. per cent respectively, azide administration resulted in corresponding values of 257, 5.05, and 97 mg. per cent 110 minutes later. 40 units of insulin were then given intravenously and the animal died 45 minutes later, when these values were found to be 109, 12, and 189 mg. per cent respectively.

The effects of  $\text{NaN}_3$  were also studied in two rabbits which had previously been rendered diabetic by alloxan administration. When nembutal was given to such animals in the usual fashion, they became highly excited

TABLE III  
*Metabolic Effects of Azide Plus Insulin*

An adult male rabbit, weight 3700 gm., was given 30 units of insulin subcutaneously with nembutal and 60 minutes later 45 mg. of sodium azide in 20 cc. of 0.85 per cent sodium chloride.

Time	Blood glucose	Serum inorganic P	Serum organic P	Blood lactic acid	Blood pyruvic acid
min.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
0	121	4.4	0.7	39	1.4
60	93	2.8	0.9	28	1.1
120	81	1.6	1.0	75	2.4
180	77	2.9	0.9	149	2.1
235	82	6.3	1.3	254	2.8

within 3 or 4 minutes. This excitement persisted for 20 to 30 minutes, during which time the animals' movements were entirely without coordination and they completely lost the use of their hind limbs. They then lost consciousness and remained deeply anesthetized for 90 to 120 minutes, and came out of the anesthesia very slowly over the following 2 hours. In consequence the desired studies were performed on other alloxan-diabetic animals without the use of nembutal. No essential difference was observed from those already shown in Table II, except that the initial blood sugars were found to be 364 and 419 and these rose to terminal values of 536 and 632 mg. per cent respectively.

*Sodium Fluoride*—The response to this drug was quite similar to those described above and a typical protocol is shown in Table IV. The preliminary drop in inorganic phosphate was again followed by a very marked increase. The concentrations of blood glucose and lactic acid rose, while pyruvic acid and organic phosphate increased erratically and to a much

smaller extent. Spot tests for acetone bodies were consistently negative. Serum calcium determinations were performed in a few instances and these all showed a definite decrease from basal values of 10.5 to 12 mg. per cent to final values of 7 to 8 mg. per cent. Table V is a protocol describing the effects of NaF given to a rabbit which had previously been given a large dose of insulin. The effects of NaF on the serum phosphate and blood lactic acid were again manifest, but blood glucose remained below

TABLE IV  
*Metabolic Effects of Sodium Fluoride*

A male rabbit, weight 3400 gm., was given 750 mg. of NaF in 50 cc. of water subcutaneously.

Time	Blood glucose	Serum inorganic P	Serum organic P	Blood lactic acid	Blood pyruvic acid	CO <sub>2</sub> capacity
min.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	vol. per cent
0	121	5.0	0.75	21	1.3	51
60	229	2.9	1.3	97	1.6	
120	396	3.5	1.9	153	3.5	24
180	505	7.7	2.1	219	3.2	
215	580	8.9	1.8	245	3.4	
245	500	14.9	2.5	303	3.7	8

TABLE V  
*Metabolic Effects of Sodium Fluoride Plus Insulin*

A young female rabbit, weight 1700 gm., was given 20 units of insulin subcutaneously and 60 minutes later 400 mg. of NaF in 30 cc. of water subcutaneously.

Time	Blood glucose	Serum inorganic P	Serum organic P	Blood lactic acid
min.	mg. per cent	mg. per cent	mg. per cent	mg. per cent
0	117	4.7	0.4	27
60	67	3.6	0.5	19
120	66	3.0	0.9	61
180	62	5.4	1.0	104
250	60	15.2	1.2	248

normal throughout the experiment. Again, insulin given after the hyperglycemia had been established proved capable of lowering the blood sugar but did not affect the rise in either inorganic phosphate or lactic acid. All animals showed much the same gross picture of fluoride poisoning: intense, thick salivation, marked air hunger, and terminal convulsions which may perhaps be related to the diminution in blood calcium. The extent of the acidosis is indicated by the diminution in serum bicarbonate concentration.

*Sodium Iodoacetate*—Iodoacetic acid (Eastman) was recrystallized from petroleum ether and dried in a glass column through which dry air was passed (Table VI). The resultant crystals were white and gave a very faintly positive starch test for free iodine. These were dissolved in water and the pH adjusted to 7.0 before subcutaneous administration. The results obtained with this drug were more variable than those encountered in any other instance. There always occurred an increase in blood glucose and lactic acid, but these were not as marked as the results obtained with the other metabolic inhibitors. After a preliminary decrease, serum inorganic phosphate sometimes rose to values greater than normal and sometimes failed to do so. However, in no case was the terminal inorganic phosphate concentration greater than 6.8 mg. per cent. In contrast,

TABLE VI

*Metabolic Effects of Iodoacetate*

A young male rabbit, weight 2800 gm., was given 200 mg. of iodoacetic acid in 20 cc. of water, pH adjusted to 7.0.

Time	Blood glucose	Serum inorganic P	Serum organic P	Blood lactic acid	Blood pyruvic acid
min.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
0	92	3.9	0.8	25	0.9
70	107	2.4	1.3	43	1.8
125	136	3.9	1.5	53	1.4
200	189	5.4	2.7	86	1.7
270	258	6.8	3.4	126	2.0

there consistently occurred a considerable increase in serum organic phosphate. No acetone bodies were ever detected in the plasma of such animals. Convulsions were observed in only a few instances but acidotic hyperpnea was invariably seen.

*Sodium Malonate*—The lethal dose of sodium malonate is considerably greater than that of the other drugs employed. The malonic acid was dissolved in water, neutralized to pH 7.3, and injected subcutaneously as a hypertonic solution. No preliminary drop in inorganic phosphate was ever encountered, but the other measured components behaved as they had done with the other inhibitors, except that the terminal values obtained in this fashion were usually somewhat higher than those which had been previously found. Serum obtained from animals which had survived more than 2 hours invariably gave strongly positive tests for acetone bodies. A typical protocol is shown in Table VII. When doses which were fatal within 4 hours were employed, it was not found possible to prevent the marked hyperglycemia even when given to animals in insulin convulsions. In one instance, a 2800 gm. male rabbit was given 30 units

of insulin. Convulsions occurred about 90 minutes later when the blood glucose was 34 mg. per cent, serum inorganic phosphate 2.1 mg. per cent, and lactic acid 32 mg. per cent. At this point, 4 gm. of malonic acid were given. The animal died 110 minutes later at which time glucose, inorganic phosphate, and lactic acid concentrations were 154, 9.7, and 290 mg. per cent respectively. No protocol of such an experiment is given, since it would so closely resemble that in Table VII.

The succinoxidase activity of tissues from a malonic acid-poisoned rabbit was very kindly measured by Dr. Frederick Bernheim. A 2020 gm. rabbit was given 3.2 gm. of malonic acid in 50 cc. of water, pH 7.2. The animal died 145 minutes later, at which time the glucose, phosphate, and lactic acid concentrations were 252, 17.8, and 174 mg. per cent respectively. Samples of liver, kidney, and heart were dispersed in a Ringer-phosphate

TABLE VII  
*Metabolic Effects of Sodium Malonate*

A male rabbit, weight 3800 gm., was given 6 gm. of malonic acid subcutaneously in 50 cc. of solution, pH 7.3.

Time	Blood glucose	Serum inorganic P	Serum organic P	Blood lactic acid	Blood pyruvic acid	CO <sub>2</sub> capacity
min.	mg. per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent	vol. per cent
0	110	4.5	0.9	27	1.1	53
60	243	5.1	0.8	85	1.7	
150	360	5.9	1.7	124	2.0	19
190	515	16.3	2.3	174	2.9	6

medium with a small Waring blender and incubated at 37° in Warburg flasks in the presence and absence of succinate. Since the concentration of tissue usually employed for such determinations would have meant dilution of the tissue malonate below its effective inhibiting range, rather thick breis were employed in such a way that in the final reaction mixtures liver was diluted four times, kidney six times, and heart seven times. The theoretical O<sub>2</sub> uptake was 190 microliters, which was accomplished in less than 20 minutes by control preparations from normal animals. At 30 minutes the uptakes for the malonate-poisoned tissues were heart 90 microliters, kidney 74 microliters, and liver 39. At 60 minutes the uptakes were heart 160 microliters, kidney 137 microliters, and liver 86 microliters. This inhibition of the succinoxidase activity of heart muscle may be presumed to be at least a contributory cause of death in these animals. All but two of the eight animals studied died with greatly dilated hearts and in two animals which had survived for 5 and 7 hours a considerable quantity of ascitic fluid and a pleural edema were found.

A separate series of rabbits was used to study plasma creatine-creatinine



relationships under the same experimental conditions. After obtaining a normal sample of blood from the ear vein, lethal doses of azide, fluoride, and malonate were given subcutaneously. The plasma proteins were precipitated with tungstic acid. Creatinine was determined colorimetrically in one aliquot with the use of alkaline picrate. A second aliquot was brought to pH 1.5 with HCl, autoclaved for 30 minutes, cooled, neutralized, and the creatinine determined in the same fashion. The color was estimated in the Evelyn colorimeter. The difference between the two values was taken to indicate the creatine concentration. The results are given in Table VIII. In each case there occurred an appreciable increment in both creatinine and creatine. The last column expresses the amount of phosphate, as phosphorus, which may have entered the plasma from the breakdown of the quantity of creatine phosphate indicated by the incre-

TABLE VIII

*Effect of Metabolic Inhibitors on Plasma Concentrations of Creatine and Creatinine*

Inhibitor	Plasma inorganic P	Creatinine	Creatine	Maximum creatine phosphate P
	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Azide, initial .	4.7	2.7	0.5	
" final ..	12.8	4.1	3.3	1.3
Fluoride, initial .	3.5	3.7	0.8	
" final	14.1	6.2	7.1	2.7
Malonate, initial . .	4.3	3.1	0.7	
" final . . . . .	14.4	4.6	2.4	1.1

ment in plasma creatinine plus creatine. It will be seen, however, that at most this could have contributed but 20 per cent of the total increment in inorganic phosphate. No determinations of adenine nucleotide were made. However, the results suggest the possibility that the rise in inorganic phosphate originated from the breakdown of creatine phosphate and the adenine nucleotides in the peripheral tissues.

#### DISCUSSION

From the foregoing data, it appears that lethal doses of each of the carbohydrate metabolism inhibitors evoked similar responses. There occurred a marked rise in the concentrations of blood glucose and lactic acid, serum inorganic phosphate, creatine and creatinine and, usually, a considerable increase in organic phosphate and pyruvic acid as well. The nature of the organic phosphate was not determined. However, because of the complexity of carbohydrate metabolism in the intact animal, no completely adequate explanation of these data by correlation with data obtained, *in vitro*, now appears possible.

The increased blood glucose might result from either increased liver production or decreased peripheral utilization. The ability of insulin to prevent this increase probably points to the former possibility. Kaplan and Greenberg (7) have reported hyperglycemia in rats given sublethal doses of fluoride, but hypoglycemia when lethal doses were employed. These phenomena could not be duplicated, except in a few of the fasted rabbits studied. In a number of normally fed rabbits, also, blood sugar reached a maximum about 90 minutes before death and then fell slowly but remained over 200 mg. per cent. This may have been due to exhaustion of the supply of liver glycogen, or, as suggested by Kaplan and Greenberg, it may have been due to inhibition of the liver phosphatase by fluoride.<sup>1</sup> Since the latter authors employed fasting rats, and in our animals the serum inorganic phosphate continued to rise while the glucose fell, and since this final fall in blood glucose has also been observed in animals poisoned with azide and malonate which do not inhibit liver phosphatase, it does seem more likely that depletion of the liver glycogen is responsible for these phenomena. Such depletion was reported by Irving (3) in iodoacetate-treated rabbits. It is, however, noteworthy that blood glucose concentrations as high as 350 mg. per cent have been observed in rabbits which had been fasted for 4 days before administration of fluoride, azide, or malonate. The rapidity of this response suggests that when the normal progress of carbohydrate oxidations is impaired, the liver responds by elevating the blood sugar.

The increased serum inorganic phosphate might be thought to result from the hydrolysis of glucose-6-phosphate in the liver as blood glucose is liberated. But this rise has also been observed in animals in which blood glucose was maintained at subnormal levels by the action of insulin. Further, the increase in phosphate never paralleled the increase in glucose but, rather, happened largely in the last 30 or 60 minutes of the experimental period in contrast to the glucose which rose steadily. It would seem more plausible that the phosphate is derived from the breakdown of adenosine triphosphate and creatine phosphate when energy from carbohydrate oxidation is no longer available. This is indicated by the appearance of appreciable quantities of plasma creatine and would be

<sup>1</sup> Apparently, NaF inhibits only the "acid phosphatase" of liver extracts (14). If the pH of parenchymatous liver cells is of the order of 5.2 to 5.6 as found by Rous (15), then NaF would be expected to inhibit the hydrolysis of glucose-6-phosphate by liver cells or by liver extracts at a pH below 6.0. The failure of NaF to inhibit the "alkaline liver phosphatase" may account for the negative results of Cori, Cori, and Schmidt (16), who used liver extracts at pH 7.2 in contrast to the thick, cellular Latapie mince employed by Ostern, Herbert, and Holmes (17) in which glucose-6-phosphate did accumulate in the presence of NaF when glycogen or glucose-1-phosphate was added.

consistent with the creatinuria and phosphaturia observed by Milhorat *et al.* (5) in iodoacetate-poisoned dogs.

The increased creatine and creatinine of plasma were not the result of migration from the erythrocytes, since actually the creatine and creatinine concentrations in whole blood rose to almost the same extent. Further, there occurred only a slight elevation in the non-protein nitrogen of whole blood and there was no apparent uremia, although it remains possible that these drugs inhibit urea synthesis. Hence, while the appearance of plasma creatine under these circumstances appears reasonable, the increased concentration of creatinine is difficult to explain.

Respiratory distress was a consistent finding and appears to have been due to the acidosis occasioned by the accumulation of lactic acid. The latter is a reasonable finding in the anaerobic conditions imposed by cyanide, azide, and malonate. But fluoride and iodoacetate, both of which interfere with anaerobic glycolysis, also prompted high blood lactic acid values. This might be expected to happen if there occurred a greater inhibition of liver glycogenesis from blood lactic acid than of the glycolytic breakdown of muscle glycogen. It is noteworthy that insulin, in doses sufficient to maintain subnormal concentrations of glucose despite the administration of fluoride, azide, or malonate, did not prevent the accumulation of lactic acid. Thus while insulin may stimulate glycogen deposition in muscle (18) and promote the synthesis of fatty acids from glucose (19) and may also inhibit the process of liver glycogenolysis, it does not accelerate glycogenesis from lactic acid, nor do the present data suggest an actual inhibition of this process.

The increase in plasma acetoacetic acid observed in malonate poisoning again offers two possibilities. Increased liver production because of impaired liver carbohydrate oxidation would appear to be unlikely, since the other inhibitors also interfere with this process, albeit at different stages. On the other hand decreased oxidation of acetoacetic acid by interference with the di- and tricarboxylic acid cycles, which have been implicated in this process (20-22), appears to be quite reasonable.

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#### SUMMARY

The administration of cyanide, azide, malonate, fluoride, and iodoacetate in doses which were lethal within 3 to 5 hours resulted in much the same metabolic pattern. Large increases in the concentrations of blood glucose and lactate, plasma inorganic phosphate and creatine, and

smaller increments in the concentrations of blood pyruvate and plasma creatinine and organic phosphate were invariably found. Insulin prevented the rise in blood glucose but not that of the other components. Of this series only malonate induced an increase in the plasma acetoacetate concentration. The significance of these findings is discussed.

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# THE FRACTIONATION OF LECITHINS AND CEPHALINS

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The lecithins and cephalins are complex mixtures of chemical entities differing from each other (a) in their component fatty acids, (b) in the case of the cephalins, in the nitrogenous constituent, and (c) in the point of substitution of the phosphoric acid-base group in the molecule.

Power and Tutin (1) were the first to question the uniformity of the glycerophosphoric acids of lecithin. In 1912 Fourneau and Piettre (2) separated the calcium salts of the acid into two fractions and proposed that the glycerophosphoric acid of lecithin was a mixture of the  $\alpha$  and  $\beta$  acids.

Bailey (3) showed that two compounds, one a crystalline sodium salt of  $\beta$ -glycerophosphoric acid, the other an amorphous sodium salt of  $\alpha$ -glycerophosphoric acid, could be isolated from egg and brain lecithin. In 1926 Karrer and Salomon (4) presented a method for the separation of  $\alpha$ - and  $\beta$ -glycerophosphoric acid and obtained both compounds from lecithin. Finally, in a series of papers (1930-34) Suzuki, Yokoyama, and Nishimoto (5-8) reported methods for the separation of the  $\alpha$  and  $\beta$  isomeric forms of lecithin and cephalin. We have used their methods, with a few modifications, on the phospholipids obtained from the heart, liver, and brain of rats, cats, guinea pigs, and beef and recently on the phospholipids obtained from the livers of fasted mice (9).

## Method

*Phospholipid Separation*—The phospholipids were isolated from the tissues by the method of Bloor (10), with the exception that the alcohol extract of the tissue was concentrated *in vacuo*. The phospholipids were dissolved in ether, placed in the ice box overnight, and the ether-insoluble material removed by centrifugation. The phospholipids were always reprecipitated with acetone from the ether solutions to remove any adhering neutral fat and cholesterol.

*Lecithin-Cephalin Separation*—Although a quantitative separation of lecithin and cephalin was difficult to obtain through differential solubility in cold absolute alcohol, it was necessary to use this method. An ether solution of the phospholipids was transferred to a 15 ml. centrifuge tube, evaporated to a small volume, and boiled with 5 ml. of absolute alcohol.

When the volume was about 2 ml., the tube was packed in ice and put in the refrigerator overnight. It was then centrifuged cold and the absolute alcohol drawn off. 3 ml. of alcohol were then added to the precipitate and it was boiled, chilled, and again centrifuged cold.

*$\alpha$ - and  $\beta$ -Lecithin Fractionation*—The two absolute alcohol solutions of the lecithins were combined and the alcohol was evaporated. The lecithins were dissolved in as small an amount of ether as possible and placed in the ice box overnight. The ether-insoluble material (sphingomyelin) was removed by centrifugation, and the ether-soluble material was transferred to a clean centrifuge tube. A cold saturated solution of cadmium chloride in ethyl alcohol was added slowly until no further precipitate appeared, and the precipitate was removed by centrifugation. The cadmium chloride-lecithin precipitate was then washed twice with 5 ml. portions of 7:3 alcohol-ether as recommended by Yoshinaga<sup>1</sup> (11).

Acetone was added and the precipitate was thoroughly mixed with it by means of a glass boiling rod (12). The tube was placed in a beaker of hot water and heated gently to boiling for 2 minutes. The glass rod was removed and washed with a very small amount of acetone, and the tube was centrifuged. The clear acetone solution which contained the  $\beta$ -lecithin-cadmium chloride complex (soluble in hot acetone) was transferred to another tube. The precipitate which contained the insoluble  $\alpha$  compound was extracted twice more with hot acetone.

The  $\alpha$  complex precipitate was dissolved in a mixture of chloroform and 80 per cent alcohol and the cadmium was removed by the methyl alcoholic ammonia procedure of Levene and Rolf (13).

The acetone solutions of the  $\beta$  complex were combined, evaporated down to about 10 ml. with the aid of a boiling rod, and the cadmium similarly removed (13).

The solvents were evaporated from the two "isomers" and each was redissolved in chloroform. Aliquots were then taken for determination of the amount of phospholipid present by the Bloor oxidation method (14).

*$\alpha$ - and  $\beta$ -Cephalin Fractionation*—The cephalin precipitate obtained from the absolute alcohol procedure was dissolved in as small an amount of ether as possible and placed in the ice box overnight. The ether-insoluble material was removed by centrifugation. The ether solution was transferred to another centrifuge tube and a saturated solution of basic lead acetate was added slowly until no further precipitate was formed. The precipitate obtained by centrifugation was washed with 7:3 alcohol-ether; then benzene-alcohol 1:1 was added and the complex and benzene-alcohol

<sup>1</sup> The number of washings and amount of solution to use depended on the size of the sample. Two washings with 5 ml. portions were used for samples with about 15 mg. of phospholipid.

mixture were stirred for at least 3 minutes with a boiling rod (12). The tube was then placed in a water bath and the benzene-alcohol was heated gently to boiling with the aid of the boiling rod, allowed to boil for 3 minutes, and then centrifuged. The complex was thus separated into the  $\alpha$  series soluble in benzene-alcohol and the  $\beta$  series insoluble in benzene-alcohol. The  $\beta$  series precipitate was extracted twice more with the benzene-alcohol mixture.

The benzene-alcohol solutions were combined, evaporated down to dryness, the residue was redissolved in chloroform, and the solution transferred to a separatory funnel. Small portions of 1 per cent hydrochloric acid were added and the solution was washed until the acid water no longer had any detectable lead in it.

The  $\beta$  complex was dissolved as completely as possible in chloroform (a few drops of 95 per cent alcohol aided in dissolving the lipid), transferred to a separatory funnel, and washed with 1 per cent hydrochloric acid.

The chloroform solutions were each evaporated down to a suitable volume and aliquots taken for determination of the amount of lipid present by the Bloor oxidative method (14).

#### DISCUSSION

It has not yet been shown that this method of fractionation gives a pure  $\alpha$  or a pure  $\beta$  compound. To test the fractionation we have used the Karrer and Salomon method (4), which depends on the fact that the barium salt of  $\beta$ -glycerophosphoric acid when treated with barium nitrate forms a crystalline double salt,  $(\text{CH}_2\text{OH} \cdot \text{CH}(\text{OPO}_3\text{Ba}) \cdot \text{CH}_2\text{OH})_2 \cdot \text{Ba}(\text{NO}_3)_2$ , that is practically insoluble in water, whereas the barium salt of  $\alpha$ -glycerophosphoric acid does not form an insoluble double salt.

The  $\alpha$  and  $\beta$  isomers obtained by our method of fractionation were hydrolyzed with 10 per cent barium hydroxide on the steam bath under a reflux for 6 hours. The barium soaps were filtered off and the barium glycerophosphates were precipitated from the filtrates with absolute alcohol. The precipitates were dissolved in water and the excess barium was precipitated as carbonate and removed by filtration.

The filtrates were concentrated by evaporation *in vacuo* and the barium glycerophosphates were again recovered by precipitation with absolute alcohol. These barium salts were dissolved in water and a solution of barium nitrate was added. The amount of barium nitrate added depended upon the size of the sample; the original Karrer and Salomon (4) proportion of 0.8 gm. of barium nitrate in 10 ml. of water for 1 gm. of barium salt in 10 ml. of  $\text{H}_2\text{O}$  was employed.

In most cases, the barium glycerophosphates obtained from the  $\beta$  isomers formed crystalline double salt compounds after 12 hours, indicating that



$\beta$ -glycerophosphoric acid was present. From most of the  $\alpha$  compounds only barium  $\alpha$ -glycerophosphate could be recovered; as a rule the glycerophosphate showed no trace of turbidity when treated with barium nitrate. The only crystals found in these solutions were found to be excess barium nitrate.

Kay (15) has criticized the Karrer and Salomon method, since they used supersaturated solutions from which a salt could crystallize on standing without addition of barium nitrate, but we did not find this to hold true with our samples.

There was some excess barium nitrate in the  $\beta$ -glycerophosphate barium nitrate double compound, but not enough to account for all of the crystals obtained. Therefore, the procedure seems valid. Rae (16) has shown

TABLE I  
*Fractionation Data on Normal Animal Tissues*

Data, for which the extreme values in each series are given, are expressed as percentages of the total phospholipid except where indicated otherwise.

Tissue	Animal	No. of animals	Phospholipid fractionation			
			$\alpha$ -Cephalin	$\beta$ -Cephalin	$\alpha$ -Lecithin	$\beta$ -Lecithin
Liver	Beef	5	39.0-41.2*	48.2-50.0*		
	Rat	8	9.8-10.7	25.8-31.0	29.9-31.3	13.5-15.6
	Guinea pig	13	11.9-13.1	23.9-26.6	26.9-32.3	19.1-20.1
Heart	Beef	5	28.8-29.7*	57.3-63.0*		
	Cat	4	9.6-10.7	23.1-24.0	39.9-43.1	21.4-22.0
Brain	Rat	8	19.8-21.2	11.6-14.2	33.1-37.0	20.9-23.2
	Beef	5	58.6-60.2*	27.9-30.1*	70.9-75.0*	19.9-21.5*

\* Expressed as the percentage of lecithin or of cephalin sample.

that migration of the phosphate from the  $\alpha$  to  $\beta$  position or vice versa does not occur during the hydrolysis of the phospholipid with barium hydroxide.

The lecithin isomers appeared to be free of cadmium, but most of the cephalin isomers still contained some lead which often was troublesome. The lead complex still present often made solution in chloroform difficult; so that a few drops of 95 per cent alcohol had to be added to get solutions on which Bloor oxidations could be carried out. (The hydrochloric acid procedure, therefore, does not remove all the lead, and another method should be found.)

Using this fractionation procedure we found results (Table I) in agreement with those of other workers. We found, as did Yoshinaga (11), that in liver and heart  $\beta$ -cephalin and  $\alpha$ -lecithin predominate. Our results for brain phospholipids agree with those of Rae (16), who found with an enzymatic method that in brain the  $\alpha$  forms were present in greater amounts.

## SUMMARY

A procedure is presented for the fractionation of the  $\alpha$  and  $\beta$  isomeric forms of lecithin and cephalin.

By use of this method it was found that in brain the  $\alpha$  forms predominate and in the heart and liver  $\alpha$ -lecithin and  $\beta$ -cephalin predominate.

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# A SIMPLIFIED PREPARATION OF CARDIOLIPIN, WITH A NOTE ON PURIFICATION OF LECITHIN FOR SEROLOGIC USE

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Since the original report of the isolation of cardiolipin (1), the usefulness of this substance in serodiagnostic tests for syphilis has been demonstrated (2-5). In this laboratory an antigen consisting of a mixture of cardiolipin, lecithin, and cholesterol (4) is now in routine use in complement fixation tests for evidence of syphilis (6). It was therefore important to devise more efficient and economical procedures for the preparation of cardiolipin. Some improvements over the original method have already been reported (7, 8). It has now proved possible to improve the preparation still further. The fundamental procedures are the same as those previously employed, but some steps which at first appeared necessary have been omitted, others have been simplified, and the yield has been increased by establishing the necessary conditions for complete recovery of cardiolipin from the barium salts.

The simplified procedure is presented in this paper by describing the details of a typical preparation. The method depends on the fact that cardiolipin is an acid and readily forms stable salts: the barium, sodium, and cadmium salts are those used for purification. These salts are rather easily converted one to another and the accompanying impurities may be removed in successive steps by taking advantage of the differing solubilities of the several salts in various combinations of solvents.

Since purified lecithin is also required in the mixtures used as antigens, some notes on the application of the previously described method for the purification of lecithin (9) are also included.

## EXPERIMENTAL

Five fresh beef hearts were defatted and ground in a meat chopper. The minced tissue, 5250 gm., was extracted twice with acetone for 24 hour periods at room temperature; for each extraction 1.2 ml. of acetone per gm. of tissue were used.<sup>1</sup> The acetone extracts were filtered off by suction.

<sup>1</sup> The larger proportion of acetone originally used (1) for the second extraction has proved unnecessary. The acetone extraction was previously carried out in the cold but room temperature extraction has been found equally satisfactory.

After the second acetone extraction the tissue was dried before a fan for about 4 hours, when the odor of acetone was no longer noticeable, and was then ground to a fine powder in a corn mill. The dried tissue powder weighed 1080 gm. It was extracted three times with 95 per cent methyl alcohol (U. S. P.) in the proportion of 2 liters of solvent to 300 gm. of tissue (7), each extraction being continued for a week at room temperature with frequent shaking. After the third extraction the tissue was washed on the filter with methyl alcohol and discarded.

Each extract was precipitated with 20 per cent aqueous  $\text{BaCl}_2$  (7) as soon as it was collected, and stored at  $3-6^\circ$ . After the third extract had been precipitated and allowed to stand overnight in the cold, the precipitates from all three extracts were pooled, collected by centrifugation, and washed once with methyl alcohol.

The methyl alcoholic supernatant from the first extract, after the removal of the insoluble barium salts, was precipitated with  $\text{CdCl}_2^2$  for the isolation of lecithin (see below). Some lecithin can also be obtained from the later extracts but the amount is only about 10 per cent of that from the first extract.

#### *Purification of Cardiolipin*

Sodium sulfate was used to convert the crude barium salts to sodium salts (8). To the packed precipitate in each centrifuge cup were added 4 times its volume of ether and about 50 ml. of 5 per cent  $\text{Na}_2\text{SO}_4$  for each 100 ml. of ether. The material had been evenly distributed in four 250 ml. centrifuge cups so that the volume of precipitate in each cup was about 30 ml.; this avoids the necessity for transferring to another container in order to add the required proportion of ether and  $\text{Na}_2\text{SO}_4$ . The solid was thoroughly broken up with a spatula and the whole mixed by vigorous stirring; two liquid layers separated and a bulky semisolid precipitate settled. The latter was removed by centrifugation and the liquid layers transferred to a separatory funnel.

The precipitate which separates at this point contains much organic matter as well as  $\text{BaSO}_4$ . A portion of this is water-soluble, but there is also a considerable amount of unidentified material which forms colloidal solutions in ether, from which it is precipitated by relatively small proportions of ethyl or methyl alcohol (8); hence two clear ethereal solutions of the crude material may form a precipitate on mixing, if one happens to contain a larger proportion of methyl alcohol than the other. Quantitative removal of the colloidal impurities at this stage is difficult and is moreover not essential, since such removal is readily effected at the next stage, when the crude sodium salts are dissolved in methyl alcohol. It is impor-

<sup>2</sup> Aqueous solution, approximately 50 per cent by weight.

tant, however, to reextract the precipitate in order to avoid possible losses of cardiolipin. This was accomplished by vigorously stirring the pooled precipitates with 100 ml. of ether, 50 ml. of 5 per cent  $\text{Na}_2\text{SO}_4$ , and about 15 ml. of methyl alcohol; the mixture was centrifuged, the liquid layers added to the material in the separatory funnel, and the final precipitate discarded.

The bright yellow aqueous layer was discarded and the ethereal solution washed twice with 5 per cent  $\text{Na}_2\text{SO}_4$ . This evidently removed some water-soluble impurities, as the aqueous layers were slightly yellow; the washing also serves to remove excess methyl alcohol, which if present interferes with the drying of the ethereal solution. An emulsion formed in the second washing but was readily broken by dilution with fresh ether and the addition of a little alcohol.

The ethereal solution was dried overnight on anhydrous  $\text{Na}_2\text{SO}_4$ . The solution was cloudy, and it had previously been found that the colloidal impurities interfered with filtration at this point; hence the  $\text{Na}_2\text{SO}_4$  was separated and washed by decantation. The ethereal solution was concentrated by distillation and was finally taken to dryness *in vacuo*.<sup>3</sup>

To the dried residue about 75 ml. of acetone were added and the mixture was warmed in a water bath at  $50^\circ$  with vigorous shaking. The acetone was decanted and the residue was extracted once more with acetone in the same manner. The acetone extract contained 3.5 gm. of yellow oil, which was discarded.

The acetone-insoluble fraction was dissolved in 100 ml. of ether and the cloudy solution was poured into 1 liter of methyl alcohol with rapid mixing. A finely flocculent precipitate separated, which was washed twice with warm methyl alcohol and discarded. The pooled solution and washings, about 1400 ml., were concentrated to 1 liter *in vacuo* to remove ether. The methyl alcoholic solution of crude sodium salts was designated Fraction Me-1.

*Preparation of Barium Cardiolipin*—To the solution of Fraction Me-1 was added 2 per cent of its volume of saturated  $\text{NaCl}$  (1) and the mixture was placed at  $3-6^\circ$  overnight. A sticky precipitate settled, from which the clear supernatant was separated by decantation. To this supernatant 20 per cent aqueous  $\text{BaCl}_2$  was added until no further precipitate formed and the mixture was chilled in ice. Vigorous shaking of the chilled mixture helped to flocculate the barium salt. The precipitate was allowed to stand overnight in the cold, then collected by centrifugation, washed once with methyl alcohol and once with acetone, and mixed with 30 ml. of ether (c.p. anhydrous) in which it formed a thick suspension. To this were added 30 ml. of acetone, and the flocculent precipitate was centrifuged and mixed

<sup>3</sup> Carbon dioxide was used to displace air in all vacuum distillations and at every point where evaporation to dryness or nearly to dryness was required.

with 40 ml. of ether, in which it now formed the stiff gel characteristic of barium cardiolipin (7). The deep yellow gel was precipitated by gradual addition of 40 ml. of acetone, and the ether-acetone precipitation was repeated three times more in the same manner. At the fifth precipitation both precipitate and supernatant appeared nearly colorless. The precipitate was then mixed with 20 ml. of ether to which a few drops of water were added; in the wet ether, the gelatinous salt gradually dissolved to a clear solution. To the light yellow ethereal solution 40 ml. of acetone were added rapidly with continuous mixing, when the salt separated as a finely divided solid.

The final precipitation from wet ether has been found preferable to the chloroform-alcohol method previously used (7). Although the precipitation from anhydrous ether by acetone removes the greater proportion of the impurities present, the efficiency of this process decreases rapidly; as the barium salt is purified, it becomes less readily dispersible in ether, and the gel tends to form lumps. This difficulty is overcome by making the final precipitation from true solution rather than from the gelatinous state.

A second crop of cardiolipin was obtained from the material precipitated from Fraction Me-1 by saturated NaCl. This precipitate, Fraction Me-2, was first redissolved in methyl alcohol; most of it dissolved readily on slight warming, and the remainder was taken up in a little ether and the ethereal solution poured into 6 volumes of methyl alcohol. The solution of Fraction Me-2 was acidified and the free acids fractionated with acetone as previously described (7), except that, in drying the free acid fraction by evaporation with absolute alcohol, only two successive additions of alcohol were made instead of five. The barium salt prepared by neutralization of the acetone-soluble acid was purified by precipitation from ether with acetone in the same manner as the first crop.

*Conversion to Sodium Salt*—As was previously reported (7), the barium and cadmium salts of cardiolipin may under certain conditions be quantitatively converted to the sodium salt by shaking their ethereal solutions with half saturated NaCl. It has now been found that the completeness of this reaction is dependent on the presence of alcohol, which presumably acts to facilitate the mixing of the reactants in the two slightly miscible liquid phases. Either ethyl or methyl alcohol may be used. In the previous method, when the barium salt had been precipitated from chloroform by alcohol and the alcohol-moist precipitate was then dissolved in ether and treated with NaCl, the necessary conditions were fulfilled and the reaction proceeded successfully, although the effect of the alcohol in the mixture was not known. When the chloroform-alcohol step was omitted, however, the importance of this factor promptly became evident, as the following experiment illustrates.

A sample of barium cardioliipin which had been precipitated by acetone and then dissolved in wet ether was shaken three times with half saturated NaCl, with the addition of only enough alcohol to break emulsions. When the ethereal solution was concentrated and poured into absolute alcohol, the recovered material was largely alcohol-insoluble and the insoluble portion proved to consist of unchanged barium salt. The recovered barium salt was redissolved in ether containing 10 per cent of its volume of alcohol and again shaken three times with half saturated NaCl; 10 ml. of alcohol per 100 ml. of ether were added to the ether layer before each addition of NaCl. After this treatment the cardioliipin was quantitatively recovered in the form of its sodium salt.

It was recently noted (8) that the crude barium precipitate from the original extracts had not been completely converted to sodium salts by the method formerly in use (7). In that method the crude salts had been washed with acetone before dissolving in ether, so that the methyl alcohol originally present was removed; it now seems probable that the incomplete reaction observed was largely due to the fact that insufficient alcohol was present.

The barium salt, purified by ether-acetone precipitation as described above, was dissolved in wet ether containing 10 per cent of its volume of alcohol and the solution was vigorously shaken for 5 minutes with one-third its volume of half saturated NaCl. The layers separated readily with no emulsification. The NaCl treatment was repeated twice, with the addition of alcohol each time in the proportion of 10 ml. for each 100 ml. of ether, and the ethereal solution was then washed twice more with NaCl to remove most of the added alcohol. The aqueous extracts were tested for the presence of barium by adding a little  $\text{Na}_2\text{SO}_4$ ; the first three extracts all gave marked  $\text{BaSO}_4$  precipitates, the fourth a faint cloud, and the fifth none. The ethereal solution of the sodium salt was dried overnight on anhydrous  $\text{Na}_2\text{SO}_4$ , filtered, concentrated by distillation to about 30 ml., and poured into 10 volumes of absolute alcohol with rapid mixing. The cloudy solution was concentrated *in vacuo* to about 80 per cent of its original volume to remove ether. During distillation a slight flocculent precipitate separated. The solution was clarified by centrifugation and placed at 3-6°; at this temperature a further trace of insoluble matter slowly separated as a film adhering to the glass. The solution had a very faint yellow color. The sodium salt at this stage of purification was designated Fraction Ba-1.

While no precise determination of the solubility of sodium cardioliipin in absolute alcohol has been made, it is of the order of 10 mg. per ml. at 3-6°. Since the salt prepared as described above is nearly pure cardioliipin, formation of more than a trace of precipitate when the ethereal solution is poured into absolute alcohol indicates that the amount of alcohol is insuffi-



cient for complete solution of the cardiolipin salt, and such precipitates should be redissolved in a little ether and poured into fresh absolute alcohol. The use of ether to disperse the material in the alcohol is advantageous because sodium cardiolipin dissolves rather slowly in alcohol alone, and some material might be lost by incomplete solution. The use of absolute alcohol facilitates the removal of the traces of insoluble impurities.

The first crop of barium cardiolipin yielded 3.0 gm. of the sodium salt, while the second crop, obtained by the acid-acetone method from Fraction Me-2, was 2.4 gm. The total, 5.4 gm. or 29.3 per cent of the original Fraction Me-1, represents a yield of 1.0 gm. of Fraction Ba-1 per kilo of minced heart muscle. The average yield from four preparations was 1.0 gm. per kilo.

TABLE I

*First Fractionation of Crude Sodium Salts, Fraction Me-1*

All samples were analyzed after removing Ba. Aliquots of the solutions were dried to constant weight at 80° in vacuo after displacing air with N<sub>2</sub>, and the weights so found were corrected for NaCl (1).

Fraction	Yield	Percent of Me-1	N	P	Iodine No
	<i>gm</i>		<i>per cent</i>	<i>per cent</i>	
Me-1	18.4	100	0.56	3.89	106
Me-2	7.9	12.9	0.45	3.68	112
Ba-1	3.0	16.3	0.05	4.05	126
Ba-2	3.3	17.9	0.53	3.70	99.8
Ba-3	1.4	7.6	1.48	3.85	112

Iodine numbers were determined by Yasuda's micro modification of the Rosenmund-Kuhnhehn method (10). Phosphorus was determined gravimetrically, essentially by the method of Elek (11), with certain modifications to be described elsewhere (12). For most of the analyses reported in this paper, the author is indebted to Miss Frances Gillum.

The results of the first fractionation of Fraction Me-1 are presented in Table I. Fraction Ba-2 was the material contained in the ether-acetone supernatants from purification of the first crop of barium cardiolipin; Fraction Ba-3, the filtrate from the precipitation of Fraction Me-1 with BaCl<sub>2</sub>. Both were freed from barium in the same way as the cardiolipin fraction.

Fraction Ba-2, dissolved in methyl alcohol, was examined for cardiolipin by successive precipitation with NaCl and BaCl<sub>2</sub> in the same way as the original Fraction Me-1, but only 0.2 gm. of barium cardiolipin was obtained. This may vary considerably, however; in other preparations from 10 to 20 per cent of the total yield of cardiolipin has been obtained by re-working Fraction Ba-2 (8).

*Final Purification of Cadmium Cardiolipin*—The previous method of purifying the cadmium salt by precipitation from benzene with ethyl acetate (1) sometimes yields the precipitate in the form of a voluminous semigelatinous mass, which retains much solvent and is almost impossible to pack down by centrifugation. A search for a substitute procedure showed that precipitation from ether by acetone gives as good results as the benzene-ethyl acetate method, with much greater ease of manipulation.

To the alcoholic solution of Fraction Ba-1, 50 per cent aqueous  $\text{CdCl}_2$  was added until no further precipitate formed, and the mixture was refrigerated overnight. The precipitate was collected by centrifugation and washed once with absolute alcohol, and the volume of the precipitate was estimated at 20 ml. by measurement of a water blank in a similar centrifuge cup. The alcohol-moist precipitate was mixed with 20 ml. of ether and the thick suspension was centrifuged. This procedure does not result in significant losses of cardiolipin, as the cadmium salt is not appreciably soluble in the mixture of solvents present, approximately equal parts of ether and alcohol. In some preparations, when a noticeable amount of color was still present in Fraction Ba-1, it was observed that most of the color was removed in the ether-alcohol supernatant from this first washing of the cadmium salt.

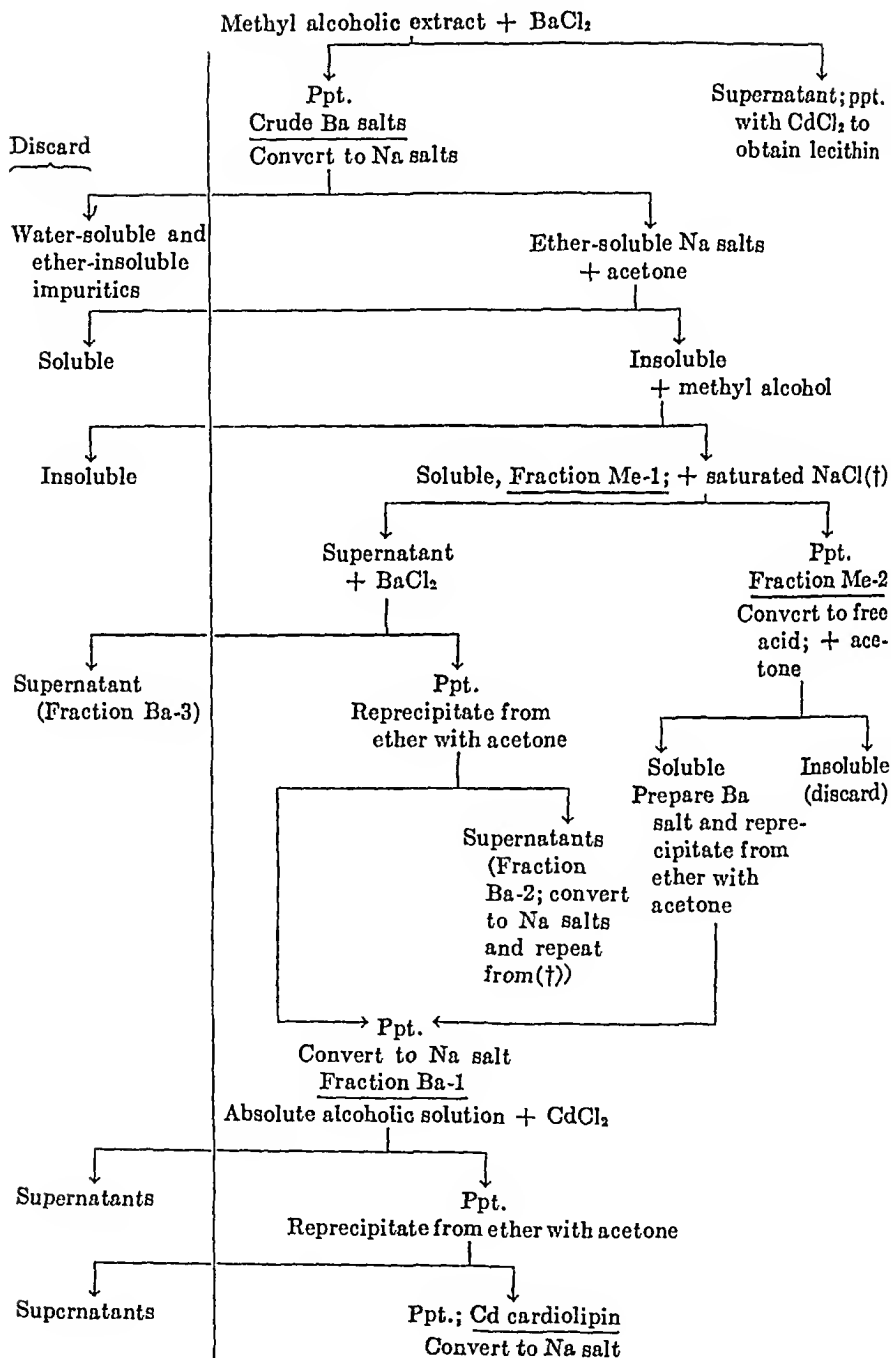
The cadmium salt was next treated with 40 ml. of ether, in which it formed a stiff gel; the gel was precipitated by the gradual addition of 40 ml. of acetone in the same manner as the barium salt, and this precipitation was repeated. The cadmium salt was next dissolved in 20 ml. of wet ether and to the clear faintly yellowish solution 60 ml. of acetone were added with rapid mixing; the finely flocculent precipitate was centrifuged and once more precipitated from wet ether in the same manner. On redissolving in ether it was now completely colorless. It was converted to the sodium salt by the method described above and the purified sodium salt was dissolved in absolute alcohol. The yield of pure sodium cardiolipin was 4 gm. or 0.76 gm. per kilo of minced tissue. No nitrogen could be detected in the purified product. The iodine number was 126.

The method of preparation is presented in condensed form in the accompanying diagram.

### *Lecithin*

No essential changes have been found necessary in the method for the purification of lecithin (9) but some modifications in the details of its application have proved advantageous.

In the method as originally described, the cadmium precipitate was washed several times with ether before fractionating with petroleum ether

*Preparation of Cardiolipin*

and 80 per cent alcohol. The treatment with ether serves to remove the greater part of the cephalin and other impurities, but it also removes considerable lecithin. Since there does not seem to be any advantage in the preliminary ether treatment, it has been omitted and the purification of the cadmium salt carried out by the petroleum ether-80 per cent alcohol method alone. It is worth noting that when the methyl alcoholic extract is first precipitated with  $\text{BaCl}_2$  to obtain cardiolipin, a large proportion of the cephalin and other substances which would otherwise be carried down with the lecithin fraction is removed in the barium precipitate; hence the supernatant from the barium precipitation yields the lecithin-cadmium salt in a more easily purified state than when the extract was initially precipitated with  $\text{CdCl}_2$  (1).

For convenient reference the material remaining in the petroleum ether layer after extraction of the lecithin-cadmium salt is designated Fraction P. Examination of this fraction reveals that there is apparently some dissociation of the lecithin-cadmium salt during prolonged extraction: after the extraction has reached an apparent end-point, Fraction P is found to contain free lecithin, which may be precipitated with more  $\text{CdCl}_2$ . This phenomenon was studied on purified lecithin. The sample, 44 gm. of lecithin which had already been twice purified over the cadmium salt, was reprecipitated from 2.4 per cent solution in alcohol with  $\text{CdCl}_2$ . The precipitate was suspended in 400 ml. of petroleum ether and extracted twenty times with 100 ml. portions of 80 per cent alcohol; the twentieth extract gave no precipitate on testing a small sample (9). Fraction P was concentrated by distillation and diluted with 95 per cent alcohol; the slightly cloudy solution, containing 6 gm. of dissolved material, gave a further precipitate with  $\text{CdCl}_2$ . This precipitate was redissolved in petroleum ether and again extracted fifteen times with 80 per cent alcohol; the final Fraction P now amounted to only 0.5 gm. but this again contained free lecithin, as its solution in alcohol gave a precipitate with  $\text{CdCl}_2$ .

From these results it is evident that the end-point of extraction, *i.e.*, the point at which a sample of the 80 per cent alcoholic extract no longer gives a precipitate on testing, is not the point at which a maximum yield of lecithin is obtained. The decision whether to rework Fraction P will evidently depend on the importance of securing a high yield of lecithin. A scheme of purification which has been found convenient and practical for routine preparation of beef heart lecithin is as follows.

The lecithin-cadmium salt, obtained from the first methyl alcoholic extract after removal of the barium precipitate, is first purified by twenty-five to thirty extractions from petroleum ether with 80 per cent alcohol; Fraction P is concentrated, diluted with alcohol, and reprecipitated with  $\text{CdCl}_2$ ; this precipitate is purified in the same way as the first lot and the two lots of partly purified cadmium salt are combined.

The salt so purified is freed from cadmium and the lecithin is recovered by complete evaporation of the chloroform *in vacuo*, then dissolved in anhydrous ether, chilled at 3-6° overnight, and centrifuged in the cold to remove the ether-insoluble fraction, which usually amounts to about 10 per cent of the lecithin. This insoluble material has not been further examined.

The ethereal solution is concentrated and the lecithin dissolved in alcohol to make a 2 or 3 per cent solution, which is then reprecipitated with  $\text{CdCl}_2$  and the precipitate purified as before. The lecithin obtained at this point, that is, after two purifications over the cadmium salt, is analytically satisfactory but usually still has a trace of yellow color. An absolutely colorless preparation of lecithin may easily be obtained if after the second purification the cadmium salt is once more resuspended in petroleum ether and extracted with 80 per cent alcohol. Cadmium is then removed as usual and the lecithin dissolved in absolute alcohol.

Lecithin purified twice over the cadmium salt, either with or without an intervening removal of cadmium and reprecipitation, has been used extensively as a component of antigen solutions and has been found satisfactory. In comparing such material with samples which had been subjected to a third purification, no significant differences could be detected either by serologic tests or chemical analysis. The amount of pigmented material removed by the third purification is extremely small. It has been noticed, however, that the faintly yellow solutions obtained after only two purifications may sometimes darken slowly on standing, whereas the water-white solutions of thrice purified lecithin remain colorless on prolonged storage in the cold (up to at least 8 months). Moreover, one of the important advantages to be derived from the use of purified phospholipids for antigens lies in their complete reproducibility; it seems advisable therefore to adhere to a rather rigid standard of purity for such materials. Consequently lecithin for serologic use is now being purified three times over the cadmium salt, to the point where no color can be detected in concentrated solutions. The yield of such thrice purified lecithin from beef hearts is about 2 gm. per kilo of minced heart tissue.

#### DISCUSSION

In preparing antigens for serodiagnostic use (3-5) mixtures of cardiolipin and lecithin in alcohol are prepared by dilution of alcoholic stock solutions, the concentration of which must be accurately determined. While it is possible to determine dry weight directly by evaporation of aliquots as mentioned above (Table I), such determinations have been found rather unsatisfactory in routine practice. In addition to the necessity of correcting for inorganic salts, difficulties in direct weighing are encountered, due to the fact that the phospholipids are hygroscopic as well as susceptible to

oxidation. For routine use it has proved most satisfactory to standardize the solutions on the basis of their phosphorus content.

The justification for this procedure obviously rests on the assumption that the materials are of uniform purity. Experience indicates that this assumption may safely be made, if the methods of purification here described are rigorously followed. Certain observations made during the preparation of cardiolipin may be employed, after a little experience, as criteria of a presumably satisfactory product: (1) the barium and cadmium salts must have exhibited the characteristic gelatinous appearance in anhydrous ether; (2) most of the color should have been removed during the purification of the barium salt, and a concentrated ethereal solution of the finally purified cadmium salt should appear completely colorless; and (3) in preparing the final absolute alcoholic solution of the sodium salt, not more than traces of alcohol-insoluble material should be found. When the concentration of this final solution is calculated from the P content, assuming 4.18 per cent of P in sodium cardiolipin, the N content of the total solids so calculated must not be greater than 0.05 per cent and the iodine number should be 115 or higher; usually it is within the range 118 to 122. *The value 4.18 per cent P is an average of analyses on eight different preparations on which careful dry weight determinations were made.* The concentration of solutions of purified lecithin may be similarly calculated, assuming that P = 3.95 per cent.

A number of different preparations of cardiolipin and lecithin prepared and standardized by these methods have been employed in serologic tests by both flocculation (3) and complement fixation (4) procedures, and in no case has evidence of qualitative or quantitative differences among the various lots been detected. The residual NaCl does not appear to affect the serologic tests, and no attempt has been made to remove it in routine preparations.

It may be pointed out that other applications will probably be found for the extremely simple method given for the interconversion of phospholipid salts. Other salts, such as the potassium salt, could presumably be prepared in the same way as the sodium salt here described. The secondary fractions obtained in the preparation of cardiolipin are readily freed from barium or cadmium by the reaction with NaCl; one can expect therefore that the method would be generally applicable in the study of salts of acidic phospholipids, such as phosphatidyl serine. The specific conditions described, *i.e.*, three extractions with NaCl in the presence of alcohol, apply to solutions containing approximately 3 gm. or less of the barium or cadmium salt in 100 ml. of ether; in working with more concentrated solutions, it might be necessary to extract with more than three portions of NaCl in order to carry the reaction to completion.

## SUMMARY

A simplified procedure for the preparation of cardiolipin is described. The purification of lecithin and the standardization of pure phospholipid solutions for the preparation of antigens are discussed.

A method is given for the interconversion of various salts of acidic phospholipids.

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# A METHOD FOR THE DETERMINATION OF DESOXYRIBONUCLEIC ACID, RIBONUCLEIC ACID, AND PHOSPHOPROTEINS IN ANIMAL TISSUES\*

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The methods used at present for the determination of the higher nucleic acids in organs are based on certain color reactions of their carbohydrate components. The quantitative evaluation of these color tests gives satisfactory results with the free carbohydrates and with the purine nucleosides and nucleotides. The application of the color tests to the higher nucleic acids, however, encounters some serious difficulties which arise mainly from the resistance of the pyrimidine nucleotides against hydrolyzing agents,<sup>1</sup> from the instability of desoxyribose, and from the necessity of separating the nucleic acid from the proteins. An extensive discussion of these problems has recently been given by Davidson and Waymouth (3). It occurred to us that these difficulties might be avoided if the quantitative estimations of desoxyribonucleic acid and of ribonucleic acid could be based on phosphorus determinations rather than on color tests of their carbohydrate components. The prerequisite for such a method would be the possibility of separating quantitatively the phosphorus fraction of ribonucleic acid from that of desoxyribonucleic acid. The separation of both P fractions can be achieved by the selective destruction of ribonucleic acid under the influence of a mild treatment with alkali. Steudel and Peiser (4)

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<sup>1</sup> Cori and Cori (1) reported recently that the color produced by yeast nucleic acid in Mejbaum's (2) test corresponded only to 40 per cent of the amount of ribose as calculated on the basis of the tetranucleotide formula. In order to avoid this difficulty, Davidson and Waymouth (3) suggested the use of ribonucleic acid instead of ribose or certain ribose mononucleotides as a standard substance for the colorimetric estimation of ribonucleic acid in tissues. This is not advisable for theoretical and practical reasons. It is very probable that the proportions of purine and pyrimidine nucleotides are different in ribonucleic acids of different biological materials. Furthermore, the only ribonucleic acid which is easily accessible is yeast nucleic acid in the form of its commercial preparations. In our experience, different batches of commercial yeast nucleic acid are not identical in regard to the mutual proportions of their component nucleotides. Even the purification of commercial yeast nucleic acid by repeated precipitations with glacial acetic acid does not lead to preparations of reliable composition.



reported the important observation that ribonucleic acid is quantitatively split into acid-soluble nucleotides when it is incubated for 24 hours at room temperature in an approximately 3 per cent solution of sodium hydroxide. Under these conditions, desoxyribonucleic acid retains its property of being insoluble in mineral acids.

Phosphoproteins differ from both nucleic acids in their behavior towards alkali inasmuch as their phosphorus groups are quantitatively liberated as inorganic phosphate during the incubation with alkali under the conditions just described. Thus, when a mixture of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins is treated with dilute alkali and subsequently precipitated with a strong acid, the total P in the precipitate represents that of the desoxyribonucleic acid, the organic P in the filtrate that of the ribonucleic acid, and the inorganic P in the filtrate that of the phosphoproteins.

#### EXPERIMENTAL

##### *Procedure for Determination of Desoxyribonucleic Acid, Ribonucleic Acid, and Phosphoproteins*

*Removal of Acid-Soluble and Lipid P*—The finely minced organ (0.5 to 5 gm.) is weighed analytically and suspended in approximately 20 volumes of ice-cold 7 per cent trichloroacetic acid. The suspension is stirred mechanically for 20 minutes and filtered on a relatively large Büchner funnel over a thin layer of Hyflo filter aid. The filtration proceeds rapidly if a sufficiently large Büchner funnel is used. The filter aid not only expedites the filtration, but in addition greatly facilitates the removal of the extracted material from the filter paper. (The centrifugation of tissue suspensions in trichloroacetic acid is not advisable because the particles do not pack well and have a tendency to float.) The residue is generously washed with an ice-cold, 1 per cent solution of trichloroacetic acid until the filtrate is free of inorganic phosphate. The washings are continued with water until the filtrate is only weakly acid towards litmus, and finally with alcohol and ether.

The residue is suspended in 30 to 40 volumes (of the wet tissue) of a mixture of 75 volumes of alcohol and 25 volumes of ether and boiled for a few minutes. After filtration and washing with ether, the dry residue is finely ground in a mortar and refluxed for 30 minutes with 30 to 40 volumes of a boiling mixture of equal volumes of methanol and chloroform. The residue is filtered on a Büchner funnel and generously washed with ether. Finally, it is dried in an evacuated desiccator.

*Treatment of Extracted Tissue Powder with Alkali and Determination of P Fractions*—The powder is quantitatively transferred to a test-tube. After addition of an exactly measured amount of  $N$  potassium hydroxide

(10 cc. per gm. of fresh tissue), the tube is closed with a rubber stopper and the mixture is incubated for at least 15 hours at 37°. Under these conditions, the tissues (except bone) are completely dissolved. After the incubation, the filter aid is centrifuged off and the total P is determined in a suitable aliquot (1 to 2 cc.) of the solution according to the method of Fiske and Subbarow (5).

For the determinations of the acid-soluble and inorganic P, another aliquot (5 cc.) is pipetted into a test-tube and precipitated by addition of 0.2 volume (of the aliquot) of 6 N hydrochloric acid and 1 volume (of the aliquot) of 5 per cent trichloroacetic acid. The precipitate contains the total amount of the desoxyribonucleic acid, while the phosphorus-containing hydrolysis products of the ribonucleic acid and the phosphoproteins remain quantitatively in the filtrate. (a) An aliquot of the filtrate serves for the determination of the total P according to Fiske and Subbarow (5). (b) In a second aliquot, the inorganic phosphate is precipitated according to Delory (6). The washed Delory precipitate is dissolved in trichloroacetic acid. The solution is centrifuged if necessary, and the inorganic phosphate is determined according to Fiske and Subbarow (5). (The preliminary isolation of the inorganic phosphate according to Delory is necessary in order to remove protein breakdown products which would interfere with the direct determination of the phosphate according to Fiske and Subbarow.)

*Calculation*—The difference between the total phosphorus of the alkaline hydrolysate ( $T_1$ ) and the total phosphorus of the acid supernatant ( $T_2$ ) represents the phosphorus of desoxyribonucleic acid; the difference between  $T_2$  and the inorganic phosphorus represents the phosphorus of ribonucleic acid; the inorganic phosphorus represents the phosphorus of the phosphoproteins.

The total volume of the alkaline solution can be calculated from the volume of the N potassium hydroxide solution by applying a correction for the increment caused by the dissolved tissue powder. It was found experimentally that this increment amounts to 0.2 cc. for 1 gm. of fresh liver tissue.<sup>2</sup> If 10 cc. of N potassium hydroxide solution are used for the digestion of 1 gm. of wet tissue, the error caused by this change of the total volume is so small that it is sufficiently accurate to add the correction,  $\Delta$  volume = 0.2 cc. per gm. of the fresh tissue, to the volume of the potassium hydroxide solution. In special cases (skin, fat tissue) it will be necessary to

<sup>2</sup> The empirical correction is somewhat larger than the theoretical increment as calculated from the amount of the tissue proteins and the average density of proteins in solutions (133). This is due to the fact that the lipid extraction and the drying of the tissue powder at room temperature do not lead to the complete removal of the water.

determine the weight of the extracted tissue in order to calculate the correction.

In order to obtain the amount of nucleic acid, the P figures are multiplied by 10.1 for desoxyribonucleic acid and by 10.6 for ribonucleic acid: It will be preferable, however, to express the results as mg. of nucleic acid P, since it is not certain whether the proportions of the nucleotides in ribonucleic acids of different origin are identical. In addition, it is not possible to give a general conversion factor for the calculation of the amounts of phosphoproteins from the amounts of the phosphoprotein P.

*Control Experiments*—As a recovery experiment, a mixture of known amounts of desoxyribonucleic acid (prepared according to Hammarsten (7)), yeast nucleic acid, and casein was added to a suspension of minced rat liver. The suspension was analyzed according to the procedure just described. A second sample of the same liver suspension was analyzed without the addition of the mixture. The differences between the correspond-

TABLE I

*Recovery of Known Amounts of Desoxyribonucleic Acid, Ribonucleic Acid, and Casein Which Had Been Added to Liver Powder*

The results are measured in mg. of P.

	Desoxyribo- nucleic acid	Ribonucleic acid	Phosphopro- tein
A, 300 mg. liver powder without addition	0.196	1.31	0.003
B, 300 " " with added mixture	0.490	1.66	0.141
B - A	0.29	0.35	0.14
Added substance	0.27	0.34	0.15

ing figures in both samples agreed satisfactorily with the amounts of the three compounds added to the liver suspension. See Table I.

If the soluble organic phosphorus after the alkali treatment represents ribonucleic acid, the trichloroacetic acid filtrate must contain ribose and purine bases (either bound or free). In several experiments ribose and total purine determinations were carried out on aliquots of the trichloroacetic acid filtrates. The ribose determinations were carried out according to Mejbaum's (2) modification of Bial's test. For the purine determinations, the aliquots of the acid filtrate were refluxed for 2 hours in the presence of 2 per cent sulfuric acid. The purine bases were precipitated from the hot filtrate by a hot solution of silver sulfate, and the nitrogen of the washed precipitate was determined according to Kjeldahl. Without exception the amounts of purines and of pentose corresponded to approximately one-half of those equivalent to the corresponding values of the organic phosphorus. The amounts of purines found are in agreement with those calculated on the basis of the tetranucleotide formula of yeast nucleic acid. The yield of

pentose amounting to approximately 50 per cent of the total present in ribonucleic acid is explained by the fact that the pyrimidine nucleotides yield only negligible amounts of furfural in Bial's test. We found that the color obtained with 50  $\gamma$  of pure cytidylic acid (8) in Meibbaum's test corresponded only to 1 per cent of the amount of ribose actually present in the nucleotide.

*Distribution of Desoxyribonucleic Acid, Ribonucleic Acid, and Phosphoproteins in Some Animal Tissues*—Table II contains representative figures for the distribution of the three P fractions in some animal tissues. The tissues were minced and fixed in trichloroacetic acid as soon as possible after the death of the animal, but no special precautions were taken to stop the action of the cellular enzymes immediately after the organs were collected. For this reason some of the figures recorded in Table II might be somewhat

TABLE II

*Amounts of Desoxyribonucleic Acid, Ribonucleic Acid, and Phosphoproteins in Some Organs*

The results are expressed in mg. of P per 100 gm. of fresh tissue.

Organ	Desoxyribonucleic acid	Ribonucleic acid	Phosphoprotein
Thymus (calf)	145	37	Negligible
" " . .	182	56	"
Pancreas (beef)	6.5	100	"
Liver (rat)	26.0	87.3	"
" " . .	22.5	102	"
Spleen "	54.5	70.0	"
Brain "	15.1	32.6	"
Kidney "	33.5	47.0	"
Egg yolk	0	11.0	116

lower than the actual amounts of the nucleic acids or phosphoproteins present in the intact tissues. This applies especially to the results obtained with pancreas, due to the high nuclease content of the organ.

Table II includes analyses of thymus, pancreas, and egg yolk. The nucleic acids and phosphoproteins in these organs have been thoroughly studied during the earlier period of the biochemistry of the nucleic acids (9, 10). According to these investigations, desoxyribonucleic acid is abundant in the thymus gland and ribonucleic acid in the pancreas, while egg yolk contains a large amount of phosphoproteins but only very small amounts of nucleic acids. It can be seen that the results presented in Table II are in agreement with the conclusions reached by the earlier investigators on the basis of the preparative isolation of the compounds.

It is interesting to note that the amounts of ribonucleic acid in rat liver far exceed those of desoxyribonucleic acid. Davidson and Waymouth (3)

recently reported similar results in an investigation which came to our attention after the completion of our own experiments. The analytical methods used by these authors were entirely different from our procedure.

#### DISCUSSION

The method presented in this paper is exclusively based on phosphorus determinations. This offers considerable advantage in comparison with procedures in which other components of the nucleic acids, such as the purines or the carbohydrates, are used for the quantitative analysis. The determination of phosphate is more reliable than that of the organic components of the nucleic acids. Furthermore, the necessity of extracting the nucleic acids from the tissue is eliminated.

On the other side, it must be considered that the phosphorus is less specific as a constituent of the nucleic acids than the purines or the carbohydrates. The interpretation of our figures as nucleic acid or phosphoprotein phosphorus rests on the assumption that nucleic acids and phosphoproteins are the only phosphorus-containing substances present in animal tissues after the extraction of the phospholipids and the acid-soluble phosphorus compounds. This assumption has recently been questioned by Davidson and Waymouth (3), who compared in a series of experiments the amounts of total nucleic acid as calculated from total P determinations with those obtained from total purine determinations in tissues after the removal of phospholipids and acid-soluble P compounds. They claimed that in many tissues the nucleic acid values as calculated from the total purine determinations are somewhat lower than those calculated from total P determinations after the extraction of the lipid and acid-soluble P. In lung and brain the discrepancies were considerable.

It appears, however, that the technique used by Davidson and Waymouth (11) for the total purine determinations involves considerable loss of purines due to acid hydrolysis of the nucleic acid. The purine figures obtained with this technique are therefore not suitable for the calculation of the nucleic acid content of the tissues. In the experiments of these authors, the tissue powder was subjected for 6 hours to an extraction with 0.1 N hydrochloric acid in a shaker at room temperature prior to the purine determinations. The acid was changed six times. This treatment is sufficient to cause a considerable loss of purines by hydrolysis of the sensitive linkage between the purines and carbohydrates, especially in thymonucleic acid.<sup>3</sup> We investigated this possibility by treating pure thymonucleate

<sup>3</sup> The liberation of the purines under these conditions is not accompanied by the formation of acid-soluble P compounds. The P-containing breakdown products are compounds belonging to the group of thymic acids which are insoluble in dilute mineral acids.

(Hammarsten) according to the directions of Davidson and Waymouth (11) and found that the N:P ratio of the nucleic acid had decreased from 1.70 to 1.45 in the course of the treatment. This means a loss of 14.7 per cent of the total N or 22.1 per cent of the purine N. It should be emphasized that the necessary acid extraction of the tissues prior to the nucleic acid determinations should be completed as quickly as possible and with ice-cold solutions. This can be achieved only if the acid extraction is carried out on the fresh tissue. Therefore, the acid extraction must precede the extraction with lipid solvents. If this order is reversed, the complete extraction of the acid-soluble P compounds requires much more time and leads to sizable losses of nucleic acids due to hydrolysis.

#### SUMMARY

1. A method for the quantitative determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues has been presented. The method is based on the different behavior of these compounds during a mild treatment with alkali.

2. The amounts of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in some animal tissues have been reported.

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# CONJUGATED FORMS OF MYELOKENTRIC AND LYMPHOKENTRIC ACIDS\*

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The names in the title of this article are proposed for the two factors found in the urine of patients with leucemia, (1, 2) and subsequently in beef liver (3); these factors have the property of stimulating the proliferation of blood cells. The names are formed from a Greek root previously used by Coffin and van Dyke (4) for hormones of the pituitary.

Myelokentric acid, which occurs in the urine of patients with myeloid leucemia, has been shown to be a keto acid, and lymphokentric acid, which occurs in the urine of patients with lymphoid leucemia, has been shown to be a hydroxy acid, obtainable from myelokentric acid by reduction; lymphokentric acid can be oxidized to myelokentric acid. These acids are soluble in fat solvents, but not in water. Biological effects similar to those produced by myelokentric and lymphokentric acid have been obtained with water-soluble fractions prepared from acidified urine by adsorption on kaolin (5, 6) and benzoic acid (7, 8).

The present work was undertaken to determine whether myelokentric acid occurred in urine as the prosthetic group of a water-soluble conjugate, or whether the water-soluble substance and the fat-soluble myelokentric acid were totally different substances producing the same biological effects. For this purpose, we have used the crude adsorbate of Heinle *et al.* (7, 8), containing the water-soluble active material from the urine of patients with myeloid leucemia. Benzoic acid was used as the adsorbent in this procedure. The product was thoroughly extracted with acetone; this should remove any free myelokentric acid. To be sure of this, we have also extracted the adsorbate with ether. The material was then hydrolyzed by heating with acid or alkali, and the hydrolysis product was extracted with ether. If myelokentric acid has been released from a conjugate by hydrolysis, the acid fraction of the final ether extract should show biological activity.

In addition, we have purified the water-soluble material by a method which effects a 20-fold concentration, and then hydrolyzed the purified substance.

\* We are grateful for the technical assistance of Nancy E. Herb.



## EXPERIMENTAL

*Technique of Animal Testing*—The aqueous solutions were given in equal doses daily to male guinea pigs weighing  $550 \pm 80$  gm. The animals were killed 1 to 3 days after the last dose; sections were taken and examined as described previously (2).

Normal butyl succinate<sup>1</sup> was found to be an excellent solvent for the oily fractions from the ether extracts. The material was given in this solvent, 0.5 cc. being used for each animal. To make sure that the use of this solvent does not change the biological effects observed in the animals, various active and inactive fractions from our previous work (1, 3) have been tested again with this solvent; the results of the new tests have agreed with the old. The life of the animals was 3 to 4 weeks in the experiments with oily solutions.

The histological changes observed in the animals described in this paper have been the same as those described previously (2).

*Preparation of Solution of Conjugated Material*—The method of Katzman and Doisy (9) was used as modified by Gurin, Bachman, and Wilson (10), except that the procedure was conducted at room temperature and the urine was not filtered after acidification. This method involves treatment of acidified urine with the adsorbent, benzoic acid, and removal of the benzoic acid from the adsorbate by washing with acetone. The dry product, corresponding to Product A of Gurin *et al.* (10), was stored in the refrigerator. From 200 liters of urine from patients with myeloid leukemia, 55 gm. of this product were obtained. This material, A, was injected into guinea pigs in the form of an aqueous solution prepared by suspending the dry product in water, adjusting to pH 7.0<sup>2</sup> with N sodium hydroxide solution, and centrifuging. The supernatant solution is designated Product A-1 in Table I.

Alternatively, Product A was extracted three times with 5 times its weight of absolute ether at room temperature. The extract was tested on guinea pigs; it was negative, except in high doses (25 liters). The residue from this was given to guinea pigs in the form of a solution brought to pH 7 with 0.5 M phosphate buffer of pH 7.9 after purification by the borate extraction and acetone precipitation method of Hirschmann, Heinle, and Wearn (8). This is designated Product A-3 in Table I.

*Purification of Conjugated Myelokentric Acid by Extraction with Alcohol*—Product A was mixed at room temperature with 10 times its weight of 50 per cent ethanol.<sup>3</sup> The resulting sludge was brought to pH 6.0 by the

<sup>1</sup> The use of this solvent was suggested by the late Dr. W. Osler Abbott.

<sup>2</sup> The pH measurements were made throughout with a Beckman meter.

<sup>3</sup> The term "per cent" is used in our papers in the sense of gm. per 100 gm. of solution.

dropwise addition of a solution made by diluting 8.0 cc. of concentrated ammonium hydroxide to 100 cc. with 50 per cent ethanol. The mixture was allowed to stand for several hours at 10° in a refrigerator; it was then centrifuged. The extraction process was repeated four times in the same

TABLE I

*Tests with Water-Soluble Products*

Two guinea pigs were used for each test, except as noted in the last column.

Product		Dose of urine	Dose of material in aqueous solution	Duration	Result*
		liters	cc.	days	
Conjugated myelokentric acid	A-1	5	15	21	++ M.
	A-3	0.5	1.7	9	+ M. (4 guinea pigs)
	"	1	3.6	9	++ M.
	"	3	9.4	9	++ "
	"	5	2.0	9	++ "
	B-1	4	10	21	++ "
	B-2	3.5	7	25	+ to ++ M. (4 guinea pigs)
Conjugated lymphokentric acid	A-1	2.3	7.5	16	+ to ++ L.
	B-1	2, 4	2.2	11, 21	++ "
Material from Hodgkin's disease	A-1	2	12	25	++ H.
	B-1	4	8	29	++ to +++ H.
Material from monocytic leukemia	B-1	2.5	14	30	+++ "
Mixture of material from myeloid and lymphoid leukemia	A-1, mixed	7 Myeloid 7 Lymphoid	13	21	++ H.

\* The significance of the + signs, and of the letters M., L., and H., is the same as has been described previously (1-3). M. designates infiltration in the organs of the animals of the myeloid type, L. indicates infiltration of the lymphoid type, and H. is used to designate a peculiar type of response differing from either, and having a superficial resemblance to lesions found in Hodgkin's disease.

manner with the residue, with 50 per cent ethanol and adjusted to pH 6.0 only if the extract was more acid than this. The combined alcoholic extracts were precipitated by the addition of 2 volumes of commercial absolute ethanol. The mixture was allowed to stand overnight in the

refrigerator and was then centrifuged, giving a precipitate designated Product B-1.

Another product, B-2, was obtained by adjusting the alcoholic solution to pH 4.5 by the addition of a solution from 2 gm. of acetic acid made up to 100 cc. with 50 per cent ethanol, and then precipitating with 2 volumes of ethanol. From 30 liters of the urine of patients with myeloid leucemia, 360 mg. of precipitate were obtained.

A product, B-3, was made by substituting 1.25 volumes of acetone for the 2 volumes of ethanol used in making Product B-2. The extraction of Product A-3 with 50 per cent ethanol and precipitation at pH 4.3 with acetone was also carried out. This gave Product B-4.

TABLE II

*Hydrolysis of Ethanolic Mother Liquor from Purification*

Method 1; two guinea pigs for each test.

Source of material	Material hydrolyzed; ethanol mother liquor from product	Dose of urine	Result*
		<i>liters</i>	
Myeloid leucemia .	B-1	12	Negative†
“ “ .	B-4	15	“ †
“ “ . . .	“	25	++ M.
Lymphoid “ .	B-1	12	+ J.
Hodgkin's disease	“	6	+ H., ++ “
Monocytic leucemia . . .	“	2.5	+ “ ++ “

\* See the foot-note to Table I.

† These animals are designated negative although they showed slight myeloid infiltrations in one organ (spleen). All the positive animals had infiltration in the liver, and at least two other organs

*Efficiency of Purification*—The discarded fractions from the purification procedure could be tested only with difficulty.

The residue insoluble in 50 per cent alcohol at pH 6.0 was suspended in water and the mixture was adjusted to pH 7.0 with N sodium hydroxide solution; this was centrifuged. The supernatant solution gave a + myeloid response in guinea pigs in doses corresponding to 15 liters of urine and was negative in lower doses.

The alcoholic mother liquor from the precipitation of Products B-1 and B-2 and the acetone-alcohol mixture from Product B-3 could not be tested as conjugated myelokentric acid. These products were hydrolyzed on the steam bath after the addition of sufficient hydrochloric acid to make the

solution 2 N in acid. After heating for 3 hours, the mixture was cooled, diluted with water, and extracted with ether. Material from the ether solution was given to guinea pigs. The results are shown in Table II.

### *Hydrolysis Procedure*

1. *Acid Hydrolysis*—The various products described above were suspended in water and made 2 N in hydrochloric acid. The resulting solution was heated on the steam bath for 3 hours. The mixture was cooled and extracted with ether. The ether was washed with distilled water until the

TABLE III  
*Hydrolysis of Water-Soluble Products*

Two guinea pigs were used for each test.

Material hydrolyzed Product		Method No. J	Dose of urine <i>liters</i>	Result*
Conjugated myelokentric acid	A	3	8	++ M.
	A-3	2 Acid fraction	2, 5	+ "
			15	++ "
	"	2 Neutral fraction	20	Negative
	B-1	1	8	+ M.
	B-4	3	8	+ "
Conjugated lymphokentric acid	"	4	8	+ ", ++ M.
	A	2 Acid fraction	4.5	+ L., ++ L.
	"	2 Neutral fraction	4.5	Negative
	"	1	10	++ L.
	"	3	10	++ ", +++ L.
Material from Hodgkin's disease	A	1	2	++ H.
	"	1	4	++ "

\* See the foot-note to Table I.

washings were no longer acid to Congo red paper, dried over sodium sulfate, and distilled. The residue from the ether was given to guinea pigs.

2. *Fractionation of Hydrolysis Product*—The ethereal extract from Method 1 was separated into acidic and neutral material by extracting the ether with 5 per cent sodium carbonate solution. The alkaline layer was acidified and extracted with ether; this contained the acid fraction.

3. *Alkaline Hydrolysis*—The adsorbates and other Products A and B were heated in 100 cc. of 2 per cent potassium hydroxide for  $\frac{1}{2}$  hour on the steam bath. The solution was cooled, acidified to Congo red paper, and

extracted with ether. The ethereal solution was washed, dried, and distilled.

4. *Vigorous Acid Hydrolysis*—In one experiment, Product B-4 from 20 liters of urine was taken up in 10 cc. of water. After the addition of 47 cc. of 2 N sulfuric acid, the mixture was autoclaved for 1 hour at 122° and 16 pounds pressure. After cooling, it was extracted with ether. The ethereal solution was washed thoroughly with water, dried, and distilled, giving 70 mg. of residue.

The products from the ether extracts of the various hydrolysis mixtures were not soluble in water. The doses shown in Tables II and III were 30 to 100 mg. The material was diluted with normal butyl succinate to 0.5 cc. for each animal. Each dose had a volume of 0.1 cc.

*Results with Urine from Patients with Chronic Lymphoid Leucemia and Other Diseases*—The method described for the purification and hydrolysis of the conjugated myelokentric acid was extended without change to material from the urine of patients with chronic lymphoid leucemia and Hodgkin's disease. The urine of one patient with monocytic leucemia was also used. The various preparations are shown in Tables I to III.

The residue from the extraction with 50 per cent ethanol was not active except in high doses, but the alcoholic solution from the precipitation showed activity (Table II). The hydrolysis of Product A from urine in lymphoid leucemia by Method 1 was incomplete. In one experiment, active material was recovered from the aqueous hydrolysis mixture after extraction with ether by adsorbing on benzoic acid (dose, 4.5 liters of urine). The conjugated material was thus not destroyed by heating with acid, but only partially hydrolyzed.

## DISCUSSION

The purification method for the water-soluble materials, described above, is adapted from a procedure used by Gurin *et al.* (10) for the purification of gonadotropic hormone. Extraction with 50 per cent ethanol effects an enormous purification, since nearly all the crude adsorbate remains in the residue. Thus, 200 liters of urine gave 55 gm. of Product A, and only 2 gm. of Product B-2.

After the hydrolysis of the water-soluble material with acid or alkali, activity was recovered in the acid fraction of the ether-soluble hydrolysis products, suggesting that myelokentric and lymphokentric acids were responsible for the biological effect of the water-soluble materials, in which they must exist as prosthetic groups. However, there was a diminution in activity after hydrolysis; this may be caused by the presence of additional water-soluble active substances, or merely by a poor yield in the hydrolysis of the conjugates. A poor yield in the hydrolysis of conjugated

products like pregnanediol glucuronide has been reported frequently (11). It is probable that the conjugated myelokentric acid is a more complex material, since it does not dialyze (8). However, the work reported here does not eliminate the possibility that other active water-soluble substances are present as well as conjugated myelokentric acid.

The production of lesions of the "Hodgkin's type" with mixtures of myelokentric and lymphokentric acids (1, 2) has been duplicated with the water-soluble materials. This confirms the results with the extracts of the urine of patients with lymphoid leucemia in indicating the existence of a conjugated lymphokentric acid. Our purification methods, like those of Heinle *et al.*, were worked out with myelokentric acid and were then extended without change to lymphokentric acid. It is not surprising that lymphokentric acid has been found in rejected fractions, for example the alcoholic solution from the purified conjugate. Additional work is necessary with the object of finding a purification method adapted solely to the concentration of lymphokentric acid, free and conjugated.

#### SUMMARY

1. Myelokentric and lymphokentric acids, which cause the proliferation of cells of the leucopoietic system, occur in urine as the prosthetic groups of water-soluble conjugates.

2. The water-soluble conjugates have been partially purified and hydrolyzed.

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# A CONVENIENT MICROTITRATION METHOD FOR THE ESTIMATION OF AMINO ACIDS\*

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This paper presents a convenient micromethod for the estimation of amino acids, based on the reaction with ninhydrin. The ammonia formed in this reaction is determined, after elimination of the interference from ninhydrin with superoxol, by addition of saturated potassium hydroxide, aeration, and titration with standard acid.

The application of the reaction with ninhydrin is one of the most specific methods for the estimation of amino acids, as the presence of an amino group in the  $\alpha$  position to a carboxyl group is required. Van Slyke and his coworkers have studied this reaction for the determination of amino acids. They pointed out that with most amino acids carbon dioxide, ammonia, and aldehydes, among other products, are evolved, proline and hydroxyproline being exceptions. Thus, it should be possible to estimate amino acids by measurement of the carbon dioxide, ammonia, or aldehydes. They have developed the method by measurement of the carbon dioxide evolved, either manometrically or titrimetrically, the manometric method being preferred (1-4). MacFadyen (5) published a method based on the ninhydrin reaction, in which the ammonia formed is measured manometrically or titrimetrically. He stated that special precautions must be taken in order to obtain quantitative results, and, even so, low values are obtained with certain amino acids. The ammonia, if allowed to react with the ninhydrin above pH 4.0, forms a stable blue complex and cannot be quantitatively recovered. MacFadyen prevented this by carrying out the reaction at pH 2.5 or lower, and then, before adding alkali, removed the ninhydrin by precipitation with hydrogen sulfide as hydrindantin. Quantitative results were not obtained with some amino acids, notably glycine, alanine, tryptophane, proline, and hydroxyproline, indicating that the ammonia may also be bound by certain breakdown products of some amino acids besides ninhydrin itself.

We had been working on this problem apparently simultaneously with MacFadyen. Similar difficulties were experienced, but the method of solving them is quite different. The entire determination is carried out in one vessel, and there are no quantitative transfers except for the aeration.

\* Presented before the meeting of the American Society of Biological Chemists held *in absentia*, 1945 (*Federation Proc.*, 4, 92 (1945)).



The amino acids are allowed to react with ninhydrin at pH 2.5 in a micro aeration tube, and then the excess ninhydrin is destroyed by heating the solution after the addition of a few drops of 30 per cent hydrogen peroxide. The tubes are set up for aeration, and saturated KOH is added. The aeration and titration of ammonia are performed as described by Sobel, Mayer, and Gottfried (6) except that the period of aeration is 40 minutes. By this method, quantitative results were obtained on most of the eighteen amino acids tested.

*Reagents—*

Caprylic alcohol, saturated with thymol.

Ninhydrin.

Buffer solution. A 10 per cent solution of solid citrate buffer of pH 2.5, made by mixing 2.06 gm. of trisodium citrate and 19.15 gm. of citric acid (1).

Hydrogen peroxide, 30 per cent, Merck's superoxol.

Saturated KOH. To a cylinder containing water covered with a thick layer of mineral oil, KOH is added until the solution is saturated. It is allowed to stand for a few days, and the clear solution is carefully aspirated into a Pyrex bottle or flask containing a layer of mineral oil about 1 inch thick to offer protection from atmospheric CO<sub>2</sub>.

Indicator. 10 parts of 0.1 per cent bromocresol green and 1 to 2 parts of 0.1 per cent methyl red in 95 per cent alcohol.

2 per cent boric acid with indicator. 20 gm. of boric acid are diluted to a liter with distilled water, and 20 ml. of indicator solution are added.

Standard 0.0714 N hydrochloric or sulfuric acid.

*Apparatus—*

Micro aeration apparatus and capillary micro burette as described by Sobel, Mayer, and Gottfried (6).

Calibrated glass spoon to deliver 50 mg. of ninhydrin, made as shown in Fig. 4 of Van Slyke and Folch (7).

*Procedure*

1 ml. of solution containing 20 to 100  $\gamma$  of carboxyl nitrogen is transferred to an aeration tube, and 0.3 ml. of buffer and 50 mg. of ninhydrin are added. The final pH should be about 2.4 to 2.6. The solutions are mixed and the tubes are put in a boiling water bath for 10 minutes. At the end of 2 minutes heating, they are withdrawn momentarily and shaken to dissolve and distribute the ninhydrin evenly. At the end of 10 minutes, 3 drops of 30 per cent hydrogen peroxide are added, and the tubes are shaken and then placed in the boiling water bath for an additional 3 minutes. The tubes are then set up for aeration, as described in the paper of Sobel, Mayer, and Gottfried (6). 1 ml. of saturated potassium hydroxide is added to each tube, and the solutions are aerated for 40 minutes. The

ammonia is trapped in 1.5 ml. of the 2 per cent boric acid-indicator mixture, and is titrated with 0.0714 *N* hydrochloric acid with a capillary micro burette.

$\text{Ml. acid} \times 1000 = \text{micrograms amino acid nitrogen}$

#### DISCUSSION

*Reaction with Ninhydrin*—A reaction time of 10 instead of 7 minutes is given, as our ninhydrin reaction mixture contains 1.3 rather than 1 ml., as described in the Van Slyke procedure (1). 5 minutes heating gave low results. It was more convenient for the authors to add the buffer as a solution, but the original method of adding solid buffer can be followed.

*Destruction of Ninhydrin*—A method of destroying ninhydrin which would not necessitate a quantitative transfer was sought. Several ketone reagents (*i.e.* the hydrazines) were tried without good results. Potassium permanganate gave improved results, but the recovery of ammonia was not quite quantitative. Superoxol was then tried, and inasmuch as quantitative results were obtained, no other compounds were investigated.

*Aeration*—The strength of the alkali used for the aeration is of paramount importance. Saturated potassium carbonate, concentrations of sodium hydroxide up to saturated, and concentrations of potassium hydroxide less than that specified all gave yields of nitrogen less than quantitative. It is important that the saturated potassium hydroxide be protected from carbon dioxide; should appreciable amounts of potassium carbonate be formed, quantitative results will not be obtained. Apparently, the function of the alkali is not merely a simple liberation of ammonia, but may serve to break up a complex formed between ammonia and the oxidation products of ninhydrin and other materials present. The alkali alone, however, is not effective. Without prior treatment with hydrogen peroxide low yields of ammonia are obtained.

The time of aeration recommended by Sobel, Mayer, and Gottfried (6) for the micro aeration procedure was increased from 20 to 40 minutes because it was noticed that the liberation of ammonia took place at a slower rate in the present procedure.

*Interferences*—It is suggested that in applying this method in the presence of compounds rich in amide or imide groups a blank be run containing the sample, but without ninhydrin, because under the conditions of the analysis a small amount of ammonia might be split off and introduce a positive error. None of the nitrogen from the amino acids tested is liberated without the treatment with ninhydrin, except for cystine and cysteine. Here the blank was 10 to 12 per cent of the ammonia released.

#### Results

Quantitative results were obtained with most of the eighteen amino acids tested; the results are listed in Table I and compared with those ob-

tained by other methods. The average deviation from the mean was 1.3 per cent for those results in which the yields were 96 per cent of the theoretical or over (Table I). The average deviation from the mean was 2.5 per cent for the other amino acids. The over-all average deviation of the mean was 1.6 per cent. Low results were obtained with hydroxyproline, tryptophane, cysteine, and cystine. Considering their chemical structure, proline and hydroxyproline would not be expected to give quantitative results by this method. The reaction of ninhydrin is not quantitative

TABLE I

*Estimation of Individual Amino Acids by Various Methods*

The results are given in atoms of N per mole of amino acids  $\times 100$ .

Amino acid	Authors' method*	Manometric CO <sub>2</sub> method, pH 2.5 (1)†	MacFadyen NH <sub>3</sub> method (5)‡
Alanine.....	99.8	100	90
Arginine.....	99.4	100	100
Aspartic acid.....	98.8	200	98
Glutamic ".....	101.4	100	97
Glycine.....	100.2	95	86
Histidine.....	100.6	100	99
Leucine.....	99.4	100	
Lysine.....	99.5	105	100 (pH 1)
Methionine.....	96.0	100	
Phenylalanine.....	97.0	100	
Serine.....	100.0	100	
Threonine.....	98.2	100	
Tyrosine.....	97.6	100	
Valine.....	100.0	100	
Cysteine.....	75.0		
Cystine (0.5 mole).....	73.0	95	
Hydroxyproline.....	28.8	100	0
Tryptophane.....	50.0	90	34

\* The samples contained 50  $\gamma$  of amino acid nitrogen.

† The samples contained 480 to 800  $\gamma$  of amino acid nitrogen (personal communication from Dr. D. D. Van Slyke).

‡ The samples contained 1000 to 1500  $\gamma$  of amino acid nitrogen (4).

with tryptophane at pH 2.5 by the Van Slyke method (1), but our results are still lower, although higher than by the MacFadyen method (5). There is also a tendency for cystine to give low results when allowed to react with ninhydrin at pH 2.5, but here also our results are lower than by the CO<sub>2</sub> method. Methionine gives practically quantitative results, suggesting that it is possible that the low results with cystine and cysteine are due to some influence of the —SH and —S—S— linkage adjacent to the amino group. Increased time of reaction with ninhydrin did not improve the low re-

sults. Increased time of aeration did not improve the results appreciably, but increased the blank for cystine and cysteine to 15 to 16 per cent of the ammonia released.

On the whole, this method gives better results than the MacFadyen ammonia method, and in many cases as good as those obtained by the gasometric  $\text{CO}_2$  method. It is to be noted that our method was worked out on 50  $\gamma$  quantities, whereas the results reported in Table I for the Van Slyke  $\text{CO}_2$  method were worked out on at least 10 to 15 times as much carboxyl nitrogen,<sup>1</sup> and the MacFadyen method on at least 20 times as much, although the  $\text{CO}_2$  method can be applied to as little as 40  $\gamma$  of carboxyl nitrogen.

It may be mentioned that lysine, a diamino acid, and arginine, with a guanidino group, give off only their carboxyl nitrogen as ammonia in this reaction. In the case of aspartic acid, the gasometric  $\text{CO}_2$  method gives double the value, whereas the present method gives the correct value.

It is our intention to adapt this method to smaller quantities, and then to apply it to the estimation of amino acids in blood. It is simple and convenient to perform and suitable for mass analysis.

We wish to thank Dr. D. F. Robertson of Merck and Company, Inc., for samples of some of the amino acids used.

#### SUMMARY

A micromethod for the estimation of amino acids is described, whereby the  $\alpha$ -amino nitrogen is split off with ninhydrin and determined as ammonia. The interference of ninhydrin with the liberation of ammonia is eliminated with the use of superoxol and subsequent aeration with concentrated KOH. All reactions are carried out in one vessel, from which the ammonia is finally aerated over into boric acid and titrated with standard acid, with a capillary micro burette. Quantitative results were obtained with fourteen out of eighteen of the amino acids tested. The method is rapid, and a large number of determinations can be carried out simultaneously.

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<sup>1</sup> Personal communication from Dr. D. D. Van Slyke.



# THE EFFECT OF PARATHYROID EXTRACT UPON THE DISTRIBUTION, RETENTION, AND EXCRETION OF LABELED STRONTIUM\*

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The purpose of this investigation has been to determine the effect of exogenous parathyroid hormone on the distribution, retention, and excretion of radiostrontium in the normal rat, with the idea that such information should be helpful in arriving at a clearer conception of the mechanism of action of parathyroid hormone. Obviously more pertinent information should be obtained by studying the effect of parathyroid extract on the metabolism of  $\text{Ca}^{45}$ , the radioactive isotope of an element with which the physiological activity of parathyroid hormone is intimately associated. However, two handicaps restrict the use of  $\text{Ca}^{45}$  that do not apply to  $\text{Sr}^{89}$ . The yield of  $\text{Ca}^{45}$  is reported to be low (1), and according to Pecher (2) determinations of its activity are extremely time-consuming.

A close analogy in the metabolism of radioactive calcium and radioactive strontium has been shown in the tracer studies reported by Pecher (2). He found that although the retention of administered radiocalcium is higher than that of radiostrontium, the distribution of the two isotopes in the different tissues is very similar, the most striking similarity being the high uptake by the skeleton. Furthermore, Pecher has shown that intravenously or subcutaneously injected radiostrontium, like radiocalcium, is excreted in both the urine and the feces.

The data reported in this paper indicate that parathyroid extract affects the distribution, retention, and excretion of radiostrontium in the normal rat by acting on the phosphorus metabolism.

## EXPERIMENTAL

The young adult rats which were used in these experiments were reared on a diet consisting of Purina fox chow, supplemented by greens and beef heart once weekly. In the first series of experiments (Table I) the animals received the stock diet throughout the experimental periods, but in the second series of experiments (Table II) the animals were denied food during the 24 hour experimental period. In most instances the experimen-

\* A preliminary report of this work has been published (*Federation Proc.*, 3, 63 (1944)).

TABLE I  
*Effect of Parathyroid Extract upon Distribution, Retention, and Excretion of Labeled Strontium*  
 The experimental and the control animals are designated by the letters E and C, respectively.

No. of pairs	Weight of animal		Para- thyroid extract adminis- tered	Time after admini- stration of $\text{Sr}^{90}$ hrs.	Per cent* of administered radiostrontium						Per cent* of administered radiostrontium recovered							
					Blood		Femurs		Kidneys		Stomach, small intestine, and contents		Large intestine- and contents		Urine		Feces	
	E	C	E	C	E	C	E	C	E	C	E	C	E	C	E	C		
	gm.	gm.	ml.															
8	279	278	5	24	0.027	0.013	2.71	2.71	0.018	0.014	0.50	0.47	2.25	1.65	11.98†	11.66†	17.40†	18.18†
	±53	±55			±0.012	±0.003	±0.99	±0.97	±0.014	±0.009	±0.31	±0.31	±1.60	±1.45	±6.37	±5.11	±5.11	±7.51
1	228	233	5	48	0.011	0.010	4.36	4.21	0.011	0.008	0.19	0.08	0.31	1.50				
8	222	220	2 × 5	24	0.030	0.021	2.30	2.70	0.472	0.026	0.79	0.58	5.07	1.44	20.31†	11.08†	4.48†	13.79†
	±20	±25			±0.006	±0.007	±0.60	±0.72	±0.136	±0.007	±0.37	±0.23	±3.98	±0.35	±7.21	±3.03	±0.53	±1.96
2	279	276	2 × 5	72			1.79	1.79	0.242	0.003	0.19	0.07	0.45	0.25				
	±40	±34					±0.35	±0.38	±0.015	±0.002	±0.05	±0.06	±0.18	±0.01				

\* Mean per cent per gm. of tissue. The measure of variability for more than two values is the standard deviation from the mean.

† Mean values for excreta of the five animals in the group which received 15 microcuries.

tal animal and its control were litter mates of the same sex, and approximately the same weight.

Each of the first nine experimental animals (Table I) received a subcutaneous injection of 5 ml. (500 Hanson units) of parathyroid extract (Lilly). 1 hour later each experimental animal and its control received an intraperitoneal injection of 0.5 ml. of a solution of 2 mg. of Sr (as  $\text{SrCl}_2$ ) containing either approximately 3 or 15 microcuries.

The last ten experimental animals (Table I) and their controls were also treated as described above, but 24 hours before the injections each experi-

TABLE II

*Effect of Phosphate Injections on Retention of Radiostrontium*

All values are expressed as the per cent of administered radiostrontium recovered. E represents the experimental animal, C the control.

Rat No.	Weight	Treatment	Femurs per gm.	Kidney <sup>a</sup> per gm.
E-20	259	4 subcutaneous injections, hourly, 1 ml. $\text{PO}_4$ buffer;* immediately after 4th injection, 2 mg. labeled Sr intraperitoneally		0.112
C-20	242	2 mg. labeled Sr intraperitoneally		0.03S
E-21	210	20 min. before administration of 2 mg. labeled Sr, 5 ml. $\text{PO}_4$ buffer subcutaneously	2.39	0.035
C-21	209	2 mg. labeled Sr intraperitoneally	2.11	0.019
E-22	323	3 hrs. before administration of 2 mg. labeled Sr, 5 ml. of $\text{PO}_4$ buffer subcutaneously	0.73	0.041
C-22	320	2 mg. labeled Sr intraperitoneally	0.62	0.013
E-23	270	4 hrs. after administration of 2 mg. labeled Sr, 2 ml. of a $\text{PO}_4$ solution† subcutaneously	2.51	0.310
C-23	260	2 mg. labeled Sr intraperitoneally	2.17	0.029
E-24	307	3 hrs. after administration of 2 mg. labeled Sr, 5 ml. $\text{PO}_4$ buffer subcutaneously	1.19	0.211
C-24	313	2 mg. labeled Sr intraperitoneally	0.81	0.036

\* Phosphate buffer, pH 6.8, P content 1.25 mg. per ml.

† Solution of  $\text{Na}_2\text{HPO}_4$ , P content 3.5 mg. per ml.

mental animal received a subcutaneous injection of 5 ml. of parathyroid extract. At the end of the desired period, measured from the time of administration of the labeled Sr, each animal was anesthetized and sacrificed by drawing blood as completely as possible. In those instances in which sufficient blood was obtained, the determination of serum Ca was made (3), in addition to the estimation of radiostrontium.

The measurement of radioactivity in the tissue samples and excreta from the animals which received 15 microcuries was made with a Lauritsen electroscope. The preparation of the tissue samples and the collection and



preparation of the excreta for the measurement of radioactivity have been described in a previous publication (4).

The measurement of radioactivity in the tissue samples of the animals which received 3 microcuries was made with our scale-of-sixteen Geiger-Müller counter.<sup>1</sup> In the preparation of the samples, 1 ml. of an appropriately diluted acid solution of the ashed sample was transferred to a piece of photographic blotting paper (12 × 24 mm.) on a glass plate. After drying at low temperature, the paper was fastened to the plate by a layer of scotch tape. The sample was then mounted in a fixed position 1.5 mm. below the counter tube, and a sufficient number of counts recorded so that the probable error in the measurement of activity did not exceed 3 per cent. In most instances the determination of activity of the experimental sample or its control immediately followed the other. During each 4 hour period of activity measurements, the counts produced by standard samples, mounted in the same manner as the samples prepared from the various tissues, were recorded. The standards represented suitable aliquots of the labeled Sr used for the injections. From these data, after correction for background counts, the per cent of the administered radiostrontium recovered in the sample was calculated.

A constant  $\beta$ -ray source was used to check the sensitivity of the Geiger counter.<sup>2</sup>

### Results

*Blood*—24 hours after the administration of 2 mg. of Sr, containing either 3 or 15 microcuries, the mean specific content of radiostrontium in the blood of five of the eight rats (Table I) which received 5 ml. of parathyroid extract was twice the mean specific content of radiostrontium in the blood of their controls. 48 hours after the administration of 2 mg. of Sr, containing 3 microcuries, the specific content of radiostrontium in the blood of an experimental animal that received 5 ml. of parathyroid extract was approximately the same as that of its control.

Judging from the weights of the animals of the first group (Table I), and the relatively enormous single dose of parathyroid extract that must be employed to elevate the serum Ca of the rat as compared to the dog, 5 ml. of parathyroid extract may not have produced a detectable increase in the serum Ca of some of the animals. That this amount of parathyroid extract was not without some effect on the serum Ca of the animals of this group was indicated by a serum Ca of 13.1 mg. per cent in one of the

<sup>1</sup> We are highly indebted to Dr. J. R. Dunning and Mr. H. A. Glassford of the Department of Physics of Columbia University for the construction of the apparatus.

<sup>2</sup> We should like to thank Dr. R. D. Evans of the Department of Physics of the Massachusetts Institute of Technology for supplying the constant  $\beta$ -ray source.

animals, weight 246 gm., from which sufficient blood was obtained for the determination of Ca as well as radiostrontium.

In five of the animals (Table I) treated with  $2 \times 5$  ml. of parathyroid extract and 2 mg. of Sr, containing 15 microcuries, the mean specific content of radiostrontium in the blood at the 24 hour interval was approximately 30 per cent higher than that observed in the controls. The mean concentrations of serum Ca in these five animals and their controls were 16.1 and 10.5 mg. per cent, respectively.

*Distribution and Retention*—At the 24 hour interval, the femurs of six of the eight animals (Table I) treated with 5 ml. of parathyroid extract contained slightly or appreciably more radiostrontium than the femurs of their respective controls, while the femurs of the other two animals contained less radiostrontium than their controls. The mean per cent of the administered radiostrontium retained per gm. in the femurs of these eight animals and their controls was in each instance 2.71. At the 48 hour interval, the femurs of an animal that received 5 ml. of parathyroid extract contained slightly more radiostrontium than the femurs of its control.

The femurs of seven of the eight animals (Table I) which received  $2 \times 5$  ml. of parathyroid extract contained slightly or appreciably less radiostrontium at the 24 hour interval than their respective controls, while the femurs of the remaining animal of the group contained appreciably more radiostrontium than its control. The mean per cent of the administered radiostrontium retained per gm. in the femurs of these animals was 2.30 as compared with 2.70 in the femurs of the controls. At the 72 hour interval, the mean specific content of radiostrontium in the femurs of two animals which had been injected with  $2 \times 5$  ml. of parathyroid extract was the same as that of their controls.

Exceedingly small amounts of radiostrontium were detected in the skeletal muscle, liver, heart, lungs, and adrenals of several animals which received 3 microcuries, and which were sacrificed after 24 hours. From two rats, treated with 5 ml. of parathyroid extract, samples of muscle contained approximately 0.007 per cent of the administered radiostrontium per gm., and the whole livers contained approximately 0.003 per cent per gm. Amounts of radiostrontium corresponding closely to the above were found in the muscle and livers of the controls. The amounts of radiostrontium detected in the livers of four of the animals which received  $2 \times 5$  ml. of parathyroid extract were of the same order as the amounts detected in the controls, but the skeletal muscle of these four hormone-treated animals appeared to have retained considerably more radiostrontium than the skeletal muscle of the controls. The mean per cent of the radiostrontium retained per gm. in the muscle of the experimental animals was 0.037 as compared with 0.007 in the controls. In the heart, lungs, and adrenals of

these four hormone-treated animals, the mean per cent of the administered radiostrontium retained per gm. was 0.012, 0.031, and 0.039, respectively, as compared with 0.007, 0.012, and 0.023 in the corresponding tissues of the controls.

At the 24 hour interval, the amount of radiostrontium retained in the kidneys of the animals (Table I) treated with 5 ml. of parathyroid extract was small and variable, and only slightly suggestive of a greater retention than that seen in the controls. However, the effect of parathyroid extract on the retention of radiostrontium in the kidneys of the animals which received  $2 \times 5$  ml. of parathyroid extract is clearly evident. It will be noted (Table I) that at the 24 hour interval the kidneys of these animals contained from 20 to 30 times as much radiostrontium as the kidneys of the controls.

*Urinary Excretion*—The mean per cent of administered radiostrontium recovered in the urine of the animals (Table I) treated with 5 ml. of parathyroid extract is only slightly greater than the control value. On the other hand, the animals which received  $2 \times 5$  ml. of parathyroid extract excreted on the average almost twice as much radiostrontium as their controls. One animal of the group excreted less radiostrontium than its control, and as previously remarked, this animal, unlike the other animals of the group, showed a higher specific content of radiostrontium in its femurs than its control.

*Fecal Excretion*—The fecal excretion of radiostrontium by the animals (Table I) treated with 5 ml. of parathyroid extract was only slightly less than that of their controls.

The animals which received  $2 \times 5$  ml. of parathyroid extract passed very few feces, and the contents of the large intestine in some of these animals contained much more radiostrontium than was found in the large intestine of the controls. As will be observed in Table I, the mean per cent of radiostrontium excreted in the collected feces was markedly less than that of the controls. These experimental animals showed marked lethargy, anorexia, and abstinence from water during the latter part of the experimental period.

*Effect of Injected Phosphates on Retention of Radiostrontium in Femurs and Kidneys*—3 hours after the intraperitoneal injection of 2 mg. of Sr, containing 3 microcuries, the amounts of radiostrontium detected in the tissues of a rat, weight 200 gm., were in per cent per gm. as follows: blood 0.021, liver 0.049, muscle 0.060, kidneys 0.220, and femurs 4.72.

The distribution pattern presented by the above values suggested that it should be possible to influence the retention of radiostrontium by increasing the flow of inorganic phosphate into the blood shortly before or after the injection of the labeled Sr. Accordingly, inorganic phosphate was administered subcutaneously to each of five rats (Table II) before or after the usual dose of labeled Sr.

The animals were sacrificed 24 hours after the administration of the labeled Sr, and the femurs and kidneys examined for their content of radiostrontium. The femurs of Rat E-20 were not examined, but in each of the other experimental animals the specific content of radiostrontium in the femurs exceeded the control value.

The kidneys of the phosphate-injected animals (Table II) contained from 2 to 10 times the amount of radiostrontium found in the kidneys of their respective controls. The greatest retention of radiostrontium in the kidneys is seen in Rat E-23, which received 7 mg. of P in the form of a solution of  $\text{Na}_2\text{HPO}_4$ . A necrosis developed at the site of injection and may have retarded the absorption of the phosphate and thus increased its effectiveness, or the alkalinizing action of the phosphate solution or its higher content of P may have favored the retention of the radiostrontium.

#### DISCUSSION

It would seem that the inorganic P level of the blood of the phosphate-injected animals (Table II) was elevated for a short time by the extra P, and that the assimilation of radiostrontium by the bones was thereby promoted. In any case, the femurs of these animals showed a higher retention of radiostrontium than their controls. It also appears that the simultaneous passage of an excess of inorganic phosphate and radiostrontium into the kidneys of these animals led to the marked retention of radiostrontium in the kidneys of some of the animals. Smith and Elvove (5) correlated the change in the serum Ca level with the change in the serum P level in rabbits under the influence of irradiated ergosterol, and concluded that an elevation in serum P as well as serum Ca is essential for calcification in the soft tissues. In their opinion the process by which calcification occurred in the soft tissues of their animals is not essentially different from that of normal ossification.

In a previous publication (4), it was shown in young adult rats, weights 199 to 254 gm., treated with 5 ml. of parathyroid extract 1 hour before the intraperitoneal injection of labeled P, that the effect of parathyroid extract was manifested within an hour by a greater accumulation of radiophosphorus in the liver and kidneys than that seen in the controls. Furthermore during the first 8 hours of the experimental period the specific content of radiophosphorus in the femurs of these animals did not decrease more rapidly than that of the controls, despite the frequency of a greater urinary excretion of radiophosphorus. However, soon after the 8 hour interval there was apparently an accelerated movement of radiophosphorus from the bones, since at the 24 hour interval the specific content of  $\text{P}^{32}$  in the femurs of the experimental animals was about 20 per cent less than that of their controls. The results in Table I indicate that the injection of 5 ml. of parathyroid extract, 1 hour before the intraperitoneal injection of labeled Sr, although most probably increasing the movement of P into the liver and

kidneys, usually did not produce changes in the blood or in the metabolism of the bone that affected the capacity of the latter to assimilate or retain the radiostrontium.

In the animals (Table I) injected with 5 ml. of parathyroid extract 24 hours before the injection of the second dose of 5 ml. and the labeled Sr, the action of the second dose of parathyroid extract most probably increased the movement of P as well as Sr and Ca into the kidneys. In seven of the eight animals sacrificed 24 hours after the administration of the radiostrontium, there seemed to have been a lower uptake or a decreased retention of radiostrontium by the femurs, and a shift in the excretion of some of the radiostrontium from the feces to the urine. It would appear that in the first few hours following the injection of the second dose of parathyroid extract a rapid urinary excretion of Sr (and Ca) and P was accompanied by a slowly rising serum Ca. Apparently it was during this period that the simultaneous passage of an excess of phosphorus and radiostrontium into the kidneys led to the marked retention of radiostrontium in these organs. Furthermore the radiostrontium appears to have been retained in such a form in the kidneys that it slowly disappeared, presumably by removal in the urine.

A high specific content of radiostrontium in the kidneys was an invariable consequence of the administration of  $2 \times 5$  ml. of parathyroid extract, but the retention of radiostrontium in the femurs of the experimental animals (Table I) at the 24 hour interval was not always appreciably less than that of the controls. At the 24 hour interval the femurs of one experimental animal of the group contained 24 per cent more radiostrontium than the femurs of its control, and the amount of radiostrontium recovered in the urine of this animal was 21 per cent less than the amount recovered in the urine of the control. Both of these effects of parathyroid extract on the radiostrontium may be explained on the assumption that the action of the second dose of parathyroid extract, superimposed on the effects of the first dose, rapidly resulted in an increased urinary excretion of P which was closely followed by an abrupt rise in serum inorganic P. In their early work on parathyroid hormone, Collip and coworkers (6) were first to observe that, when a certain critical level in the serum Ca is reached after the administration of parathyroid extract, the serum inorganic P tends to rise abruptly. The results observed in our phosphate-injected animals (Table II) indicate that an abrupt rise in the serum inorganic P, coinciding with the presence of an excess of radiostrontium in the blood, should result in an increased uptake of radiostrontium by the bones, thus diverting to the bones some of the radiostrontium which otherwise would have been excreted during the first 24 hours.

## SUMMARY

The percentage of labeled strontium has been determined in the tissues and excreta of untreated rats and in rats treated with parathyroid extract after the intraperitoneal injection of a single dose of  $\text{SrCl}_2$ , containing radioactive strontium.

1. One injection of 5 ml. (500 Hanson units) of parathyroid extract usually produced no effect upon the retention of radiostrontium in the femurs or the soft tissues, or in the excretion of radiostrontium, that was observable 24 hours after the administration of the labeled Sr.

2. The injection of 5 ml. of parathyroid extract 24 hours before the injection of an additional 5 ml. and the labeled Sr usually resulted in (a) a decreased retention of radiostrontium in the femurs, (b) a decreased fecal excretion of radiostrontium, (c) an increased urinary excretion of radiostrontium, and (d) a marked retention of radiostrontium in the kidneys.

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# THE EFFECT OF DOSE AND NUTRITIVE STATE ON KIDNEY ARGINASE AFTER STEROID STIMULATION\*

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It has been demonstrated in this laboratory (1) that many steroid hormones increased and a few decreased the arginase activity of the kidney but did not affect that of the liver or intestine. A comparison of the results obtained with the various steroids suggested that the change in arginase activity of the kidney might be related to the rate of stimulation of kidney growth and amount of steroid absorbed.

## Method

*Pellets*—The steroids<sup>1</sup> were made into cylindrical pellets of about 14 mg., diameter 2.7 mm. and height about 2.7 mm., by means of a hand press designed in this laboratory. The amount of steroid absorbed was increased by implanting two or more pellets and decreased by mixing with cholesterol in various proportions (2, 3). The amount of steroid absorbed was determined by multiplying the decrease in pellet weight by the per cent of steroid incorporated into the pellet (3).<sup>2</sup>

*Animals*<sup>3</sup>—Mice of the highly inbred Murray-Little dba strain were castrated under ether anesthesia at 16.0 to 19.5 gm. of body weight. They were fed Purina fox chow checkers.

*Duration of Experiments*—The steroid pellets were implanted subcutaneously 30 days after castration and allowed to remain *in situ* for 30 days. Testosterone propionate was studied also at 10 days of treatment.

*Autopsy*—The mice were fasted for 24 hours and then killed by decapitation. The pellets were removed, washed in distilled water, and dried in a desiccator over calcium chloride. The kidneys were removed, weighed on a Roller Smith torsion balance, and placed in a heavy walled Pyrex

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<sup>1</sup> The steroids were generously provided by the Ciba Pharmaceutical Products, Inc., through the courtesy of Dr. E. Oppenheimer and Dr. C. R. Scholz. Many of the steroids were prepared specially for this and related studies. The testosterone propionate was provided as perandren and the 17-methyltestosterone as metandren.

<sup>2</sup> Kochakian, C. D., unpublished.

<sup>3</sup> The mice were provided by the Biological Station, Springville, New York, through the courtesy of S. G. Warner.



tube containing 5 ml. of cold redistilled water. The organs were homogenized (1) and enough water was added to give a dilution of 40 ml. of water per gm. of kidney.

*Arginase Determination*<sup>4</sup>—The arginase activity was determined by pipetting 1 ml. of the homogenized kidney tissue into the substrate mixture, described by Lightbody (4) but set at pH 9.4, and incubating for 6 hours in a water bath at 37°. The reaction was ended by adding 1 ml. of 0.3 N hydrochloric acid and placing the tube in a boiling water bath for 5 minutes. The urea was converted to ammonium carbonate by means of urease, and the ammonia aerated into 10 ml. of 2 per cent boric acid and titrated with 0.01500 N hydrochloric acid. The indicator of Ma and Zuazaga was used (5). The number of ml. of hydrochloric acid was converted to arginase units from a graph prepared according to Edlbacher and Rothler (6).

Blank determinations were made by carrying out the above procedure except for the incubation period.

### Results

*Castration*—As in the previous study (1), castration did not affect the total amount of arginase of the kidney but did increase the amount per gm. of tissue (Table I). The amount of enzyme in both groups of animals is slightly but proportionately less than that obtained previously (1).

*Dose and Chemical Structure*—Testosterone, testosterone propionate, androstanol-17( $\alpha$ )-one-3, and androstanediol-3( $\alpha$ ), 17( $\alpha$ ) produce a similar effect (Figs. 1 to 4) on arginase activity of the kidney at various dose levels. There is at first a decrease followed by a marked and uniform increase in enzyme activity. If the values for the above compounds are calculated on a mole basis, these steroids have nearly identical potencies (Fig. 5). Furthermore, the values for testosterone acetate-3-propionate-17 (*cf.* (1)) also fall in this group.

A similar type of response is obtained when testosterone propionate pellets are allowed to remain implanted for 10 days (Fig. 6).

17-Methyltestosterone (Fig. 7) and to a lesser extent 17-methylandrostanediol-3( $\alpha$ ), 17( $\alpha$ ) (Fig. 8) alter the above pattern of response to dose. The presence of the 17-methyl group in these two compounds causes an immediate increase in the arginase activity of the kidney with only a probable slight and transitory decrease at extremely low levels of steroid absorption. On the other hand, androstanediol-3( $\beta$ ), 17( $\alpha$ ), 17-methylandrostanediol-3( $\beta$ ), 17( $\alpha$ ), androstanediol-3( $\alpha$ ), 17( $\alpha$ ) acetate-3, and 17-methyl- $\Delta^5$ -androstanediol-3( $\beta$ ), 17( $\alpha$ ) prolong the phase during which there is a decrease in enzyme activity (Fig. 9).

<sup>4</sup> A detailed study of many factors that affect arginase activity has been made and will be reported in another communication.

TABLE I

*Effect of Nutritive State on Increase in Kidney Arginase after Steroid Stimulation*

Nutritive state*	Treatment	No. of mice	Fasting body weight	Steroid absorbed	Kidney	Arginase	
						Total	Per gm.
			gm.	mg.	mg.	units	units
N.	Normal	12	22.5		414	11	27
U.	"	3	13.9		222	5	21
N.	Castrated	8	20.7		265	10	38
U.	"	6	16.1		235	12	48
N.	Testosterone	4	20.8	0.37	419	17	41
U.	"	1	16.4	0.50	352	7	21
N.	"	4	22.4	1.15	507	37	79
U.	"	1	17.2	1.20	393	29	77
N.	"	7	20.6	4.34	468	64	137
U.	"	5	17.1	4.40	387	42	110
N.	"	3	22.4	16.2	543	212	390
U.	"	1	18.7	17.8	401	155	336
N.	" propionate	5	21.6	2.33	492	36	72
U.	" "	4	16.3	2.67	362	26	72
N.	17-Methyltestosterone	4	23.3	0.14	417	25	54
U.	"	1	18.0	0.12	329	11	33
N.	"	6	22.8	4.26	509	122	241
U.	"	3	17.3	3.54	409	62	159
N.	17-Methylandrostanediol-3( $\alpha$ ),17( $\alpha$ )	5	22.5	3.2	519	98	187
U.	"	2	16.0	3.8	332	65	198
N.	Androstanediol-3( $\alpha$ ),17( $\alpha$ )	3	22.1	0.25	385	8	23
U.	"	1	18.9	0.30	282	8	28
N.	" acetate-3	12	21.9	2.5	416	8	20
U.	" "	4	14.7	3.2	325	11	33

\* N. = normal, U. = undernutrition.

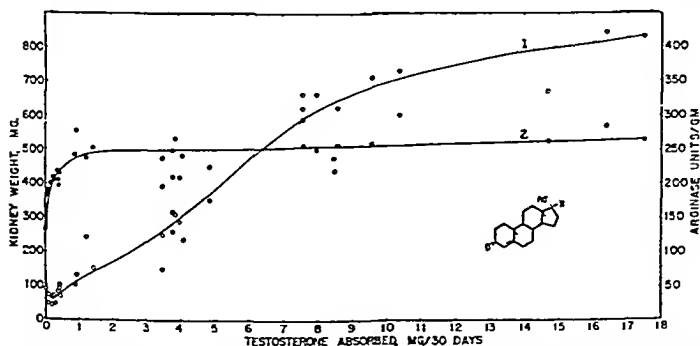


FIG. 1. The effect of the dose of testosterone on the arginase content of the kidney of the castrated mouse. The kidney of the normal mouse weighs 414 mg. and has 27 arginase units per gm. Curve 1, arginase; Curve 2, kidney.

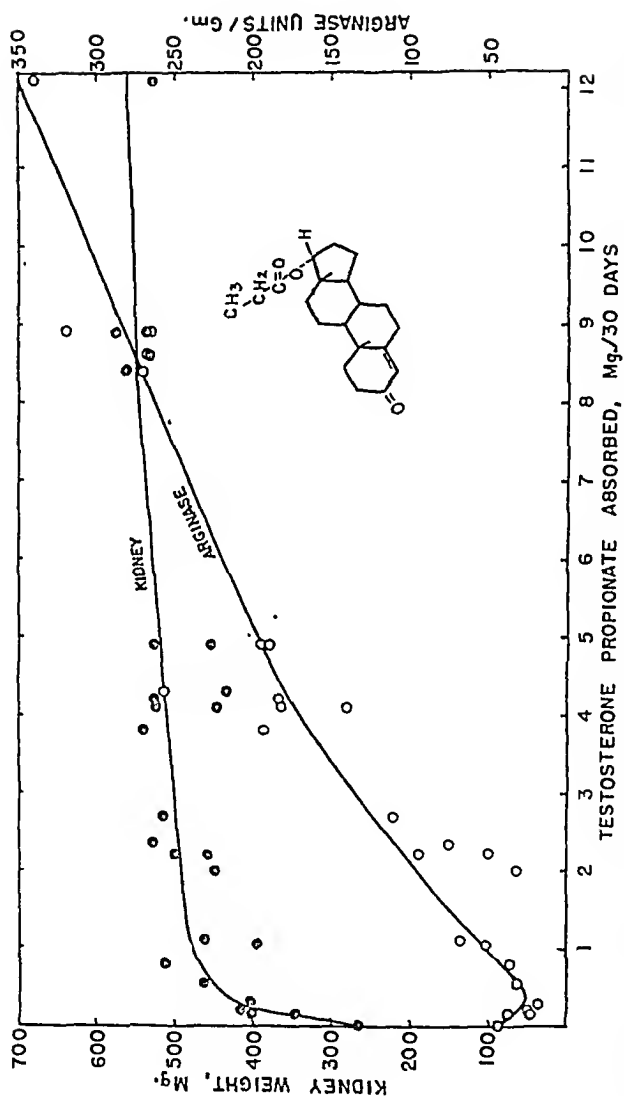


Fig. 2. The effect of the dose of testosterone propionate on the arginase content of the kidney of the castrated mouse

*Kidney Size and Arginase Activity*—The initial decrease in arginase activity corresponds to the restoration of the kidney to its normal size

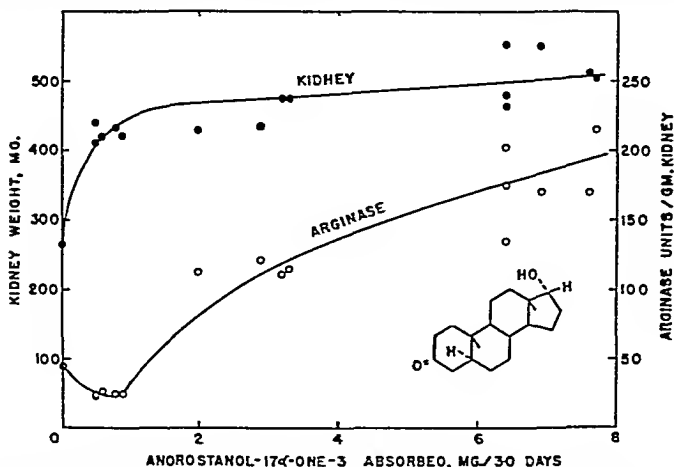


FIG. 3. The effect of the dose of androstanol-17 ( $\alpha$ )-one-3 on the arginase content of the kidney of the castrated mouse.

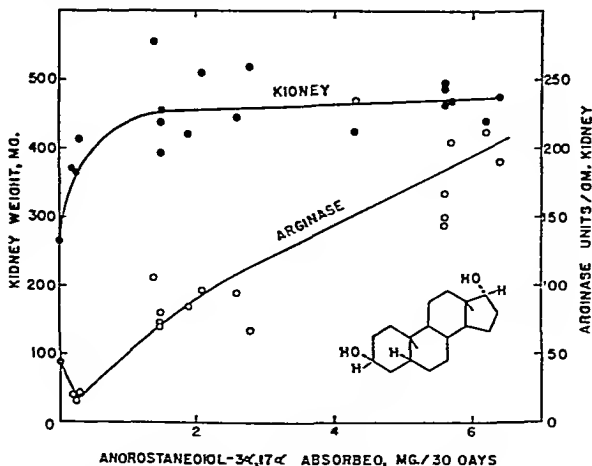


FIG. 4. The effect of the dose of androstaneol-3( $\alpha$ ),17( $\alpha$ ) on the arginase content of the kidney of the castrated mouse.

except in the case of 17-methyltestosterone, 17-methylandrostanediol-3( $\alpha$ ),17( $\alpha$ ), and the 10 day experiment with testosterone propionate (also

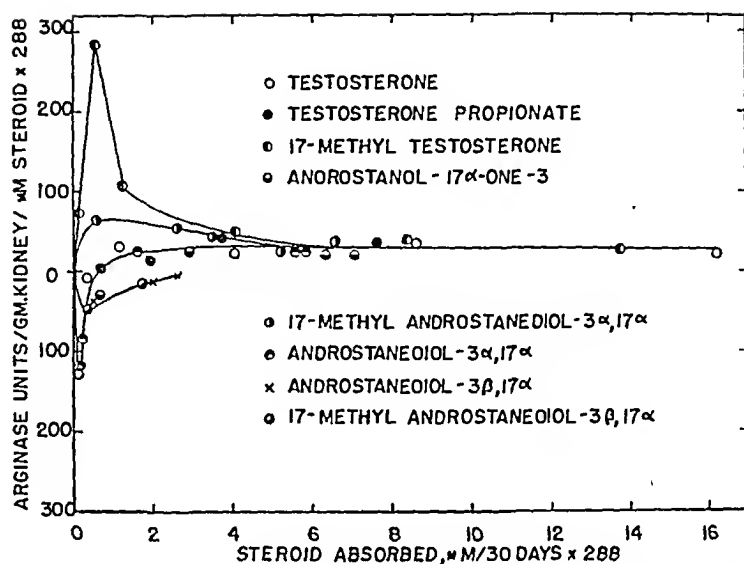


FIG. 5. A comparison of the effect of various steroid hormones on the arginase content of the kidney of the mouse. The  $\mu\text{M}$  of steroid absorbed are multiplied by the molecular weight of testosterone (288).

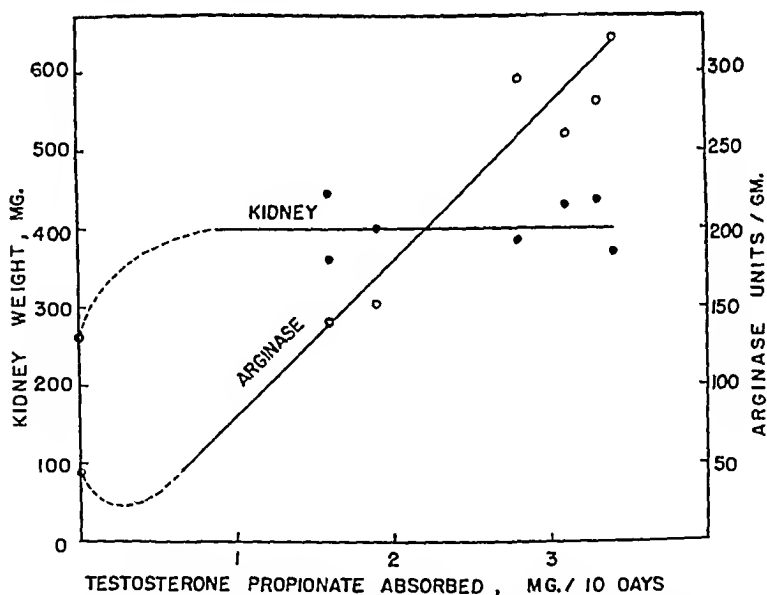


FIG. 6. The effect of the dose of testosterone propionate on the kidney arginase of the castrated mouse. These mice were treated for only 10 days. Note that the same amount of material absorbed in 10 days as in 30 days (Fig. 2) produces a greater increase in arginase without a greater increase in kidney size.

cf. (1)). In the case of these last three experiments the increase in arginase activity is evident before the normal kidney size has been exceeded.  $\alpha$ -

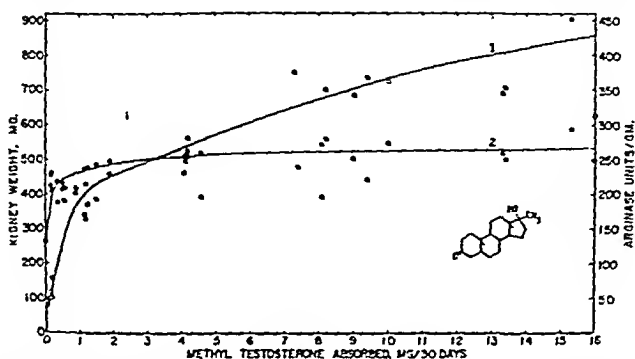


FIG. 7. The effect of the dose of 17-methyltestosterone on the kidney arginase of the castrated mouse. Curve 1, arginase; Curve 2, kidney.

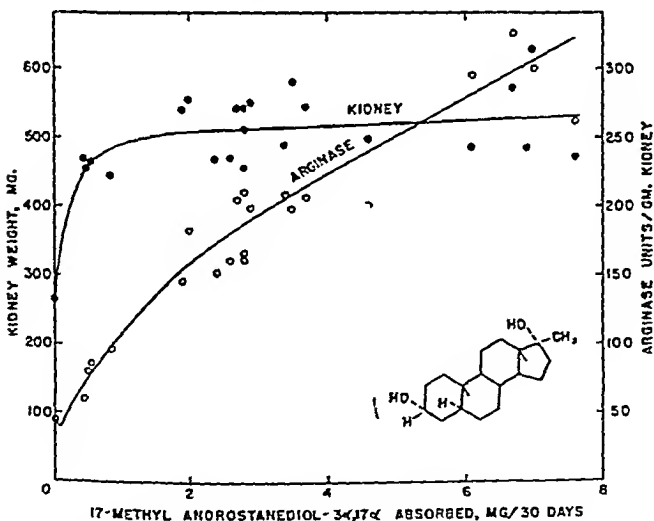


FIG. 8. The effect of the dose of 17-methylandrostanediol-3( $\alpha$ ),17( $\alpha$ ) on the kidney arginase of the castrated mouse.

Estradiol (1) also produces an increase in arginase activity without a maximum increase in kidney size. Furthermore, the increase in arginase

activity continues with increase in dose long after the kidney has reached its maximum size. Thus it seems that the steroids directly stimulate the cells to greater activity.

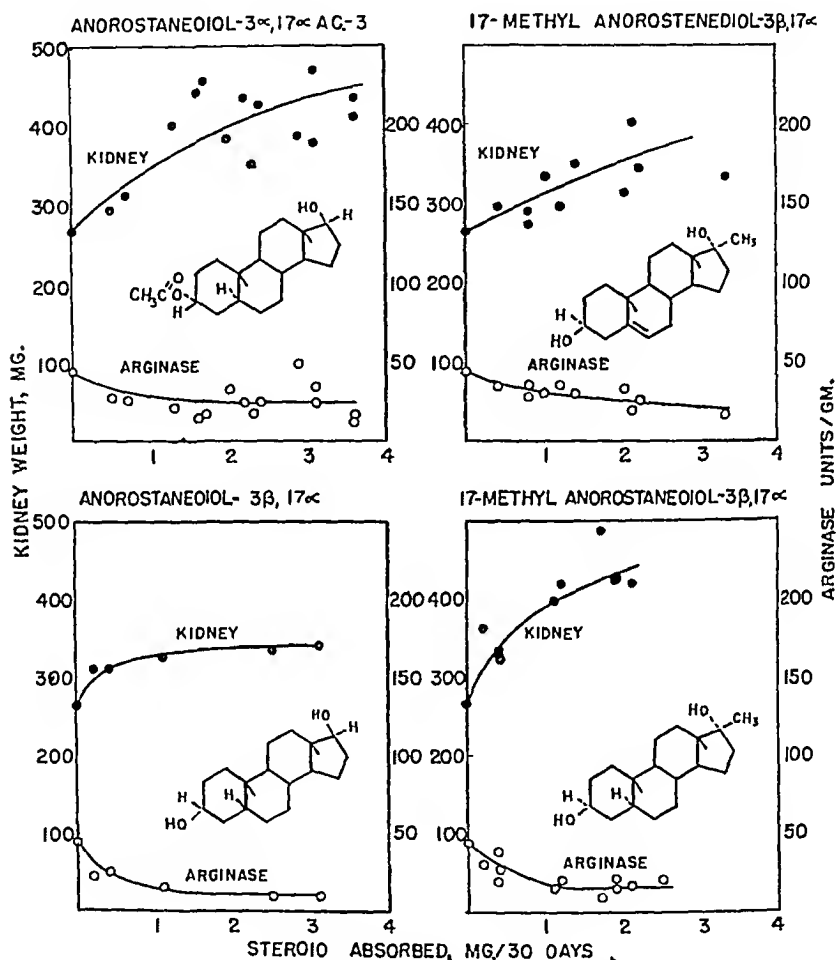


FIG. 9. The effect of less active steroids on the kidney arginase of the castrated mouse. The increase in kidney size is not accompanied by an increase in arginase because the rate of absorption of the steroids is not rapid enough to provide the necessary stimulus to the cells.

*Undernutrition and Arginase Activity*—At one time during this study, owing to technical difficulties, some of the mice did not receive the proper care, and were consequently in a state of undernutrition during most of the experimental period. The analyses of the kidneys of these animals

proved of interest and are presented in Table I. Even though there is a marked decrease in the response of the kidney weight to steroid stimulation, there is nevertheless a definite increase in the arginase activity which on a per gm. tissue basis is slightly less or as great as that occurring in the kidneys of the well fed mice. There is a smaller increase in total amount of arginase in the kidneys of the underfed mice, owing to the much smaller increase in kidney size.

The kidneys of the castrated animals and those which received ineffective amounts of steroids show no change in the total arginase but a slight and probably significant increase in arginase per gm. owing to the decreased size of the organs.

#### DISCUSSION

The study of the arginase content of the kidney at various dose levels provides further information concerning the increase in arginase activity obtained by the implantation of single pellets of the pure steroids (1). The decrease in arginase activity after administration of certain compounds (1) can be definitely attributed now to the ability of these steroids to restore the kidney only to that condition existing in the normal animal. Furthermore, the difference in arginase activity obtained among steroids which produced a maximum response in kidney size disappears when the comparison is made per mole of steroid absorbed. The kidney attains a maximum weight with increase in dose rather quickly but the arginase activity continues to increase.

In the previous report, 17-methyltestosterone and testosterone demonstrated the same ability in both 10 and 30 day experiments to stimulate an increase in kidney arginase activity. It is now apparent that these two compounds bring about their changes in a somewhat different manner. The 17-methyltestosterone causes a rapid and immediate increase in arginase activity at extremely low levels, while testosterone produces an initial decrease followed by a steady and uniform increase which eventually approaches the values produced by 17-methyltestosterone. This rapid initial increase seems to be a property of the 17-methyl group, for 17-methylandrostanediol-3( $\alpha$ ),17( $\alpha$ ) produces a similar, though not quite as marked an effect.

The continued increase in arginase activity with increase in dose long after the kidney has attained its maximum weight response indicates that the size of the organ is not a complete reflection of the rate of its cellular metabolic processes. Even in undernutrition the arginase activity per gm. of tissue is greatly increased and in proportion to the increase in kidney weight.

It is of special interest that these steroid hormones affect only the



enzymes ("alkaline" and "acid" phosphatases (7), *d*-amino acid oxidase (8), and arginase (1)) of the kidney and not those of the liver or intestine. It would seem then that the metabolic effects, *e.g.* protein anabolism (*cf.* (9)), of these compounds are mediated at least in part through the kidney. Edlbacher (10) has suggested as a result of his extensive studies on liver and yeast arginase that one of the functions of this enzyme may be to provide suitable nitrogen forms for protein synthesis.

One synthetic process, the formation of glycocyamine by the transfer of the amidine group of arginine to glycine, occurs in the kidney (11). This compound then is methylated in the liver to form creatine. Can it be that the increased arginase activity of the kidney noted after the administration of the steroid hormones is related to creatine formation? Along this same line of thought it is interesting that the 17-methyl group has an accentuating effect on both arginase and creatine metabolism (12).

#### SUMMARY

The increase in arginase activity obtained in the kidneys of castrated mice treated for 30 days with various steroids implanted subcutaneously as pellets is related to the amount and the chemical structure of the compound absorbed. There is at first a decrease in arginase activity of the kidney which occurs during the phase when the kidney is increasing in size to its maximum response. This initial phase is followed by a rapid increase in arginase activity. These two phases are altered by the chemical structure of the steroid. Testosterone, testosterone propionate, androstenediol-3( $\alpha$ ),17( $\alpha$ ), and androstanol-17( $\alpha$ )-one-3 produce almost identical responses per mole of steroid absorbed. The introduction of the 17-methyl group (17-methyltestosterone and 17-methylandrostenediol-3( $\alpha$ ),17( $\alpha$ )) causes a rapid initial increase instead of a decrease in arginase activity, which then continues at a slower rate of increase until it becomes identical with that of the former compounds.

Undernutrition does not affect the ability of the steroids to stimulate arginase activity per gm. of tissue but decreases the total increase because of the smaller increase in kidney size.

It is suggested that the increased arginase activity is related to synthetic processes such as protein anabolism and glycocyamine formation.

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# THE MICRODETERMINATION OF IODINE IN BIOLOGICAL MATERIALS WITH SPECIAL REFERENCE TO THE COMBUSTION OF SAMPLES IN THE PARR OXYGEN BOMB\*

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The most troublesome obstacle to the quantitative determination of the very minute quantities of iodine which are normally present in biological materials has been the destruction, without loss of iodine, of the relatively large amounts of organic matter present. The procedures which have been used to destroy the organic matter include fusion with alkali, combustion in a closed system, and digestion with sulfuric acid or hydrogen peroxide. Von Kolnitz and Remington (1) have critically reviewed all of these methods and point out serious objections to each.

Leipert (2) developed a wet combustion method in which the organic matter is oxidized and the iodine is converted to iodine pentoxide by means of chromium trioxide in a sulfuric acid medium. Chromium trioxide usually contains iodine and is difficult to purify. Matthews, Curtis, and Brode (3) devised a method for preparing chromium trioxide of low iodine content. The chromium trioxide method is not directly applicable to biological materials of low iodine content such as food.

For the analysis of liquid biological samples we have adapted and improved the acid permanganate method used by Riggs and Man (4) for determination of iodine in blood. Potassium permanganate is easily purified and is an excellent oxidizing agent. It serves also to indicate conveniently the amount of oxidizing agent required for complete oxidation of the organic matter, and in the distillation procedure the decoloration of the permanganate color is assurance of complete reduction of both the iodine and permanganate.

However, the permanganate method is also not applicable directly to biological samples of low iodine content, such as food, which necessitate the oxidation of considerable organic matter. For such samples some type of closed combustion is usually employed. McClendon was the first to make closed combustions on biological materials in a bomb (5), but shortly discarded the bomb for a silica combustion tube (6). The latter method finally developed to the use of a platinum combustion tube with

\*The data presented in this paper were taken from a project covered by a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

brick furnace, a platinum-tipped screw feed stoking device, and an absorber with triple sintered glass disks (7). An electrometric method is used in the titration. Karns (8) proposed ignition in oxygen in a specially constructed flask, and collection in a liquid absorption train. Von Kolnitz and Remington (1) modified Karns' technique and made a vertical screw feed of brass with a water seal. This procedure is slow and requires considerable attention by the analyst. Peel, Clark, and Wagner (9) have described a gravimetric procedure for the determination of chlorine, bromine, and iodine in organic compounds following decomposition in the Parr sodium peroxide semimicro bomb. This method is limited to samples of semimicro size of 20 to 50 mg. There is also the danger of loss due to the explosive action of sodium peroxide.

For the microdetermination of iodine in samples of high carbon content, such as food and feces, we have developed a method which employs a simple, safe, and rapid procedure for destruction of the organic matter. The procedures concerned with the liberation and determination of the iodine incorporate the best features of the methods of Riggs and Man (4) and of Matthews, Curtis, and Brode (3) and include several original modifications. This improved method consists of combustion of the sample in the Parr oxygen bomb and absorption of the iodine in dilute alkali at the bottom of the bomb, digestion of the wash solution in an acid permanganate medium, reduction and liberation of the iodine with phosphorous acid, and distillation of the iodine in an all-glass apparatus. The iodide in the distillate is oxidized by the Groák (10) permanganate procedure to iodate and titrated with sodium thiosulfate with the iodide-iodate-starch reaction.

### *Special Apparatus*

*Bomb Calorimeter*—The Parr oxygen bomb calorimeter, the single valve Illium oxygen bomb, model B, and accessories may be purchased from the Parr Instrument Company, Moline, Illinois.

*Distillation Apparatus*—This has been completely described and drawn to scale by Matthews, Curtis, and Brode (3). It may be obtained from the Leonard Glass Works, 1432 Minnesota Avenue, Columbus, Ohio.

The distillation flasks of this apparatus are used for the digestion with permanganate.

Titration are made with a Koch automatic micro burette of 1 ml. capacity and graduation interval of 0.01 ml., which has a stoppered reservoir and two interchangeable ground glass tips, one of which delivers 100 to 110 drops per ml. This may be purchased from the Arthur H. Thomas Company, West Washington Square, Philadelphia.

A Fisher fluorescent titration illuminator provides a cool glareless light that aids in detecting the delicate end-point.

*Reagents*—The potassium permanganate was recrystallized according to the directions of Riggs and Man (4). All other reagents were prepared according to the directions of Matthews, Curtis, and Brode (3).

### *Procedure*

*Combustion*—An appropriate amount of dried sample containing about 2 to 5  $\gamma$  of iodine is pelleted or packed into a combustion cup and weighed accurately. 20 ml. of  $N$  NaOH are introduced into the bottom of the bomb to capture the volatile iodine compounds. The bomb is assembled, filled with 15 atmospheres of oxygen, and the sample is ignited as usual in the determination of caloric value. 3 minutes are allowed for complete combustion and the oxygen is liberated slowly (to prevent possible loss of iodine). The contents of the bomb are transferred to a digestion flask and the combustion cup and bomb are thoroughly rinsed with 0.1  $N$  NaOH. The washings are also transferred to the digestion flask.

*Digestion*—To the washings in the flask are added several glass beads as antibumps, 2 gm. of recrystallized potassium permanganate, and 100 ml. of 18  $N$  sulfuric acid. Each flask is connected by means of a 3-hole rubber stopper with a 200° thermometer, a small stream of compressed air to prevent foaming, and a vent to carry off the fumes and vapors. The flasks are heated by means of a small gas flame and the digestion is continued until the temperature reaches 175°.

Liquid samples, which are relatively low in organic matter, can be oxidized directly by acid permanganate digestion. Such samples require more potassium permanganate, depending on the amount of organic matter present. It is important that sufficient permanganate be added so that the digestion mixture does not decolorize before a temperature of 175° is reached. If the sample should decolorize it must be discarded. The sulfuric acid must be added slowly with shaking of the flask and an occasional cooling under tap water, as a violent foaming reaction takes place.

*Distillation*—When the digest has cooled to below 100°, the thermometer is washed with approximately 20 ml. of double distilled water from a wash bottle. 80 ml. more water are added and the flask is shaken thoroughly to mix the contents. An Erlenmeyer flask containing 1 ml. of a solution 2  $M$  with respect to potassium carbonate and 0.2  $M$  with respect to sodium sulfite is placed under the condenser stem and tilted so that the tip dips into the solution. The digestion flask is connected to the distillation apparatus by means of the air-tight ground glass joint. The flask is then heated and, as soon as distillation begins, 5  $M$  phosphorous acid is added slowly through the entry tube. After the mixture in the distillation flask is completely reduced and becomes colorless, 2 ml. of phosphorous acid in excess are added to insure complete liberation of the iodine and the distillation is continued until about 100 ml. of distillate are collected.

The distillate is evaporated on a hot-plate to a volume of about 5 ml. The sample is then transferred quantitatively to a 50 ml. Erlenmeyer flask and the flask is washed three times with small volumes of water, so that the total volume is not more than 25 ml. The solution is again evaporated to about 5 ml.

*Grodk Permanganate Oxidation*—The flask is placed in a shallow boiling water bath on a hot-plate. 0.2 M potassium permanganate is added dropwise directly into the solution until a permanent purple color is obtained. The flask is then gently tilted and rotated to mix the solution, which is then heated 3 minutes. 10 drops of 8 N sulfuric acid are then added. The permanganate should not be decolorized. If decoloration does take place, more permanganate must be added immediately. After heating 3 minutes more, 1.5 M sodium nitrate solution is added dropwise directly into the sample until all manganese dioxide and excess permanganate are reduced. 1 drop of sodium nitrate in excess is added and the sides of the flask are thoroughly washed by carefully rotating the flask in order to reduce any manganese dioxide particles adhering to the sides. The solution is heated for 3 minutes more and then 5 drops of 5 M urea solution are added and the sides of the flask thoroughly washed by rotating the flask. The sample is kept in the water bath for 9 minutes longer. The sample is cooled to room temperature, then chilled on ice before titration.

*Titration*—A small crystal of potassium iodide and 2 drops of a 1 per cent starch solution are added to the sample which is then titrated with 0.0002 N sodium thiosulfate solution delivered from a 1 ml. micro burette. 1 ml. of 0.0002 N  $\text{Na}_2\text{S}_2\text{O}_3$  is equivalent to 4.23  $\gamma$  of iodine.

*Blank*—The procedure for determining blanks is essentially the same as that used for sample analysis. 100 ml. of 18 N sulfuric acid and 10 gm. of potassium permanganate are used in determining the blank. The titration value of the blank is subtracted from the titration value of the sample to give the true iodine content of the sample.

#### EXPERIMENTAL

*Recovery of Pure KI*—To determine the recovery of iodine following oxidation either by digestion with acid permanganate or by combustion in the Parr oxygen bomb the following experiment was carried out. Cellulose blocks were placed in combustion cups and moistened with 0.5 ml. of N NaOH. Then exactly 1 ml. of standard potassium iodide solution was adsorbed on the blocks which were dried in a 65° oven overnight. The samples were then oxidized either by digestion in acid permanganate or by combustion in the oxygen bomb before the acid permanganate digestion.

TABLE I

*Recovery of Standard Solution of Inorganic Iodine following Oxidation by  $\text{KMnO}_4$  Digestion or by Combustion in Oxygen Bomb*

1 ml. of KI (= 1.04 ml. of 0.0002 N  $\text{Na}_2\text{S}_2\text{O}_3$ ) pipetted on a cellulose block.

	Dried (65° oven) overnight			Not dried
	Block wet with NaOH	No NaOH	No NaOH	Block wet with NaOH
	Combustion in bomb before $\text{KMnO}_4$ digestion and distillation		$\text{KMnO}_4$ digestion and distillation	
Average titration, ml.....	1.06	1.08	1.02	1.07
" recovery, %.....	101.9	103.8	98.1	102.9

TABLE II

*Recovery of Iodine after Combustion of Sample (Feces) in Porc Oxygen Bomb*

Sample No.	Treatment	Weight of sample	Iodine in sample	Iodine per gm.
		gm.	γ	γ
1	Regular ignition in bomb	1.5946	4.86	3.05
2	" " " "	2.0525	6.39	3.11
3	" " " " + bomb shaken	2.5634	7.61	2.97
4	" " " " + $\text{O}_2$ in bomb released by bubbling into 25 ml. 0.1 N NaOH through exhaust attachment	1.0080	3.09	3.07
5	Regular ignition in bomb	1.7483	4.23	2.42
6	" " " "	1.8185	4.23	2.33
7	" " " " + $\text{O}_2$ in bomb released by bubbling into 50 ml. 0.1 N NaOH through exhaust attachment	1.8887	3.93	2.08
8	Same as Sample 7	1.8273	4.19	2.29
7A			0.17	
8A			0.17	
9	Blank		0.13	
10	"		0.17	

TABLE III

*Recovery of Iodine Added (5 Micrograms Each) to Various Biological Materials*

No. of analyses	Sample		Iodine in sample	Total iodine	Recovery of added iodine	
		ml.	γ	γ	γ	per cent
12	Pure sweat	125	1.97	6.73	4.76	95.2
7	Body washings	250	1.44	6.44	5.00	100.0
12	Urine	25	1.50	6.37	4.87	97.4



TABLE IV  
*Duplicate Analyses of Iodine in Various Biological Materials*

Material	Amount of KMnO <sub>4</sub> used	Sample size	Iodine content of sample	Iodine concen- tration
	<i>gm.</i>	<i>gm.</i>	<i>γ</i>	<i>γ per gm.</i>
Feces F665*	2	2.866	2.54	0.89
		2.961	2.50	0.84
		2.540	2.16	0.85
C631*	2	3.121	7.44	2.38
		3.262	7.87	2.41
		3.156	7.76	2.44
Food*	2	2.976	3.93	1.32
		2.759	3.38	1.23
		2.851	3.89	1.36
		2.557	3.34	1.31
		2.700	3.60	1.33
		<i>ml.</i>		<i>γ per 100 ml.</i>
Milk	22	50	2.41	4.8
		50	2.33	4.7
		50	2.45	4.9
		50	2.28	4.6
Urine D593	2	5	1.99	40
		5	2.03	40
F601		5	1.35	27
		25	6.64	27
E639		5	15.23	305
		5	15.19	304
Sweat C646	6	150	4.36	2.9
		150	4.48	3.0
E648	6	150	2.88	1.9
		150	2.88	1.9
F649		150	6.47	4.3
		150	6.39	4.3
Sweat and body washings C587	9	500	1.78	0.356
	15	750	2.71	0.361
D592	9	500	0.80	0.160
	15	750	1.23	0.164
D634	9	300	8.46	2.8
		300	8.42	2.8
Body washings B397	9	400	2.37	0.592
		400	2.37	0.592
E411		250	2.16	0.864
		250	2.24	0.896
C608		750	4.99	0.665
		750	4.99	0.665
D613		750	4.27	0.569
		750	4.19	0.559
E659		300	2.62	0.873
		300	2.58	0.860

\* Ignited in bomb.

Distillation and Groák oxidation were carried out on all samples as usual. The results are presented in Table I. These results indicate that the completeness of oxidation and recovery of potassium iodide following both methods of oxidation are of a high order. The wetting of the sample with alkali is not necessary for complete recovery of iodine.

Table II gives the data obtained in testing the procedure for possible loss of iodine during liberation of oxygen from the bomb following combustion of the sample. In Sample 4 the 25 ml. of 0.1 *N* NaOH into which was bubbled the oxygen released from the bomb were added to the regular sample, and in Samples 7 and 8 these absorption solutions (Samples 7A and 8A) were analyzed separately. The results in Table II as well as in Table I show that there is no loss of iodine in the release of oxygen from the bomb as outlined in the procedure.

Table III gives the results of recovery tests on three types of liquid biological samples, the iodine content of which was determined by the method outlined when combustion of the sample in the bomb is not necessary. The recoveries were 95.2, 97.4, and 100 per cent.

The results of duplicate determinations on various biological materials analyzed in this laboratory are presented in Table IV. The samples analyzed range in size from 1.0 gm. of dried feces (Table II) to 1 liter of sweat and body washings, and from iodine values of 1.99  $\gamma$  to 15.23  $\gamma$  per sample, and indicate the range of application of the method.

#### DISCUSSION

Riggs and Man (4) employed ceric sulfate and copper as catalysts in the determination of iodine in blood. These investigators found, however, that the results of a few analyses of blood with and without the catalysts did not differ significantly. The results obtained in our recovery tests indicate that the catalysts are not essential for the determination of iodine in the materials analyzed. Also a second digestion in dilute permanganate solution as carried out by Riggs and Man (4) was found to be unnecessary, especially when phosphorous acid was used for reduction in the distillation procedure. Liquid samples as large as 1 liter have been digested directly in acid permanganate without a preliminary evaporation.

For digestion and distillation the apparatus described by Matthews, Curtis, and Brode (3) is more compact and simple to operate than that used by Riggs and Man (4) or by Trevorrow and Fashena (11).

As already pointed out,  $\text{KMnO}_4$  is superior to chromium trioxide as an oxidizing agent, not only because of the greater ease of purifying the former but also because it serves conveniently during the distillation to indicate by decoloration when all the permanganate and iodine are reduced. An-

other objection to chromium trioxide is the formation, when the oxidation mixture is heated too long at a high temperature, of an insoluble form of chromium sulfate which causes bumping during distillation. Oxalic acid is undesirable as a reducing agent during distillations because of the evolution of a large amount of carbon dioxide which necessitates the use of a large amount of permanganate in the Groák procedure. Phosphorous acid, as prepared and used by Matthews, Curtis, and Brode (3), is much more satisfactory.

In the procedure of Riggs and Man (4) great importance is attached to the temperature and length of time of distillation. They warn that recoveries of iodine are low unless reduction takes place at a temperature above 135°, and unless distillation after the initial addition of oxalic acid is continued for at least 30 minutes and about 170 ml. of distillate are collected. In the procedure presented no attention is paid to the temperature, and the distillation of 100 ml. is sufficient to give complete recovery of iodine.

A solution 2 M with respect to potassium carbonate and 0.2 M with respect to sodium sulfite is easily prepared and is conveniently added to the receiving flask with only one pipette. This solution will keep for 3 to 4 weeks in cold storage.

The use of phosphoric acid in the Groák procedure, as observed by Riggs and Man (4), results in a slow development of the starch iodine color and erroneously high titers. Its use also leads to an indefinite yellowish end-point.

Potassium iodide solution is not stable and must be freshly prepared. The use of one to two crystals of KI is therefore preferred.

#### SUMMARY

A method is described for the determination of micro amounts of iodine in biological materials. For liquid samples the Riggs and Man procedure for determining iodine in blood has been modified. In the case of materials of high organic content, such as food, the sample is dried and ignited in the Parr oxygen bomb before digestion in acid permanganate.

This method has been successfully applied to samples ranging in size from 1 gm. of a solid sample to 1 liter of a liquid sample.

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# THE EFFECT OF ENVIRONMENTAL TEMPERATURE AND POTASSIUM IODIDE SUPPLEMENTATION ON THE EXCRETION OF IODINE BY NORMAL HUMAN SUBJECTS\*

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Iodine balance studies were carried out for the first time by von Fellenberg (1) on himself in 1926. This was possible only after his fundamental research resulted in the development of a micromethod sensitive enough to determine quantitatively the minute amounts of iodine excreted by the normal human subject. Scheffer (2, 3) studied the iodine balance in normal subjects and patients with thyroid disease. Both of these investigators recognized the importance of the skin as an avenue of excretion in attaining a true balance of iodine. Curtis and associates (4-6) also measured the dermal excretion of iodine in their extensive investigations of the effects of thyroid disease on iodine metabolism.

The dermal excretion in the above mentioned investigations has been loosely termed "sweat," although the environmental temperatures did not exceed 24° and most of the subjects were hospital patients confined to bed. Furthermore, the dermal excretion was obtained only after repeated washing of the body and underclothes, not by a collection of sensible perspiration. These results on the loss of iodine through the skin, however, suggested the importance of studying the dermal excretion of iodine under conditions which would induce profuse sweating.

## EXPERIMENTAL

The data to be reported in this paper are taken from a 2 year study of the effects of high environmental temperature and variable humidity upon the dermal losses of certain minerals and vitamins and their metabolites. During eight of the experimental weeks of this study in which the subjects were on a constant adequate diet, iodine was determined in the dermal excretions collected in 8 hour periods in a controlled environment and in the urinary excretions collected in 24 hour periods. During four of these weekly periods, iodine was also determined in the daily food

\* The data presented in this paper were taken from a project covered by a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

of each subject and in the daily fecal collections. For 4 of the 8 weeks the subjects received daily 2 mg. of KI each.

Five young men, 20 to 28 years of age, were used as subjects. For 8 hours each day, Monday through Friday, the subjects were kept in an air-conditioned room maintained on alternate weeks at "comfortable" (about 28.9° dry bulb, 50 per cent relative humidity) and "hot moist" (38.3° dry bulb, 69 per cent relative humidity) conditions.

Before entering the air-conditioned chamber each subject washed his entire body thoroughly with soap and water, then rinsed with distilled water. By means of a preboiled and rinsed piece of cheesecloth each subject removed all excess rinse water from the surface of his body. The subjects then entered the chamber with no clothing other than klaks.

While in the chamber, the subjects did no work or exercise but sat on chairs or cots which were completely covered with rubber sheeting and towels, the latter preboiled and rinsed in distilled water. Visible body sweat was absorbed on cheesecloth, preboiled and rinsed thoroughly in distilled water, and collected in a jar containing 10 cc. of glacial acetic acid. At the end of the 8 hour period each subject washed his body first with 600 cc. of distilled water. The second, third, and fourth body washings were made with 300 cc. portions of distilled water each, and these as well as five additional 300 cc. portions of distilled water were used in succession to wash and rinse cheesecloths, towels, klaks, chairs, and any other surface upon which skin excretion might have collected. All undiluted sweat was combined with the body washings for analysis.

Before entering the exposure chamber, and immediately on leaving it, the men were weighed without clothing to the nearest gm. on a Troemner balance. The final body weight plus the weight of all urinary and fecal excreta subtracted from the initial body weight plus the weight of all food and water ingested gives the "net loss" in body weight. The net loss in body weight under comfortable conditions averaged 92 gm. per hour, representing largely insensible vapor loss. Under hot moist conditions, the net loss in body weight averaged 676 gm. per hour, representing mainly the secretion of the sweat glands.

On three Saturday mornings a 4 hour collection of undiluted sweat was made under hot moist conditions for iodine analysis. On these days each subject stood in a large pan so as to collect all sweat running off the body, and also collected sweat continuously by running the lip of a clean beaker over all readily accessible parts of the body. The samples were preserved by the addition of glacial acetic acid.

The food and feces samples were dried, ground, and kept in tightly stoppered glass jars until analyzed. All samples were analyzed for iodine by the methods described in detail in the previous publication (7).

### Results

The concentration of iodine in undiluted sweat is given in Table I. Without iodine dosage, the iodine concentration in the sweat of three subjects averaged 0.95  $\gamma$  per 100 cc. A single dose of 2 mg. of KI increased the average concentration to 3.18  $\gamma$  per 100 cc. Fourteen daily doses of 2 mg. of KI, taken prior to the collection day, produced, when compared with the single 2 mg. dose, an additional increase in the iodine concentration of the sweat in the one subject whose concentration had been low. The other two subjects showed an actual decrease, which is probably of no significance.

A comparison of the hourly loss of iodine through the skin and kidneys under different environmental conditions is presented in Tables II and III. With a constant diet in Weeks 13 and 14 the dermal excretion of iodine increased, on the average, 2.3 times from comfortable to hot moist conditions. At the same time the urinary excretion of iodine showed no significant change. The average combined urinary and dermal excretion

TABLE I

*Concentration of Iodine in Undiluted Sweat with and without KI Dosage*

The results are given in micrograms per 100 cc.

Wk. No. (Saturday)	Iodine supplement before sweat collection	Subject C	Subject D	Subject E	Average
12	None	0.54	1.22	1.08	0.95
15	1 dose of 2 mg. KI	1.53	4.91	3.08	3.18
27	14 daily doses of 2 mg. KI	4.17	4.17	2.62	3.65

per hour under hot moist conditions showed an increase of 2  $\gamma$  over that under comfortable conditions, although one subject failed to show this relationship.

In Weeks 17 and 18, when the diet was supplemented daily with 2 mg. of KI, the dermal excretion of iodine under hot moist conditions averaged about 20 times that under comfortable conditions. The urinary excretion of iodine showed an average drop of 9.9  $\gamma$  per hour under the hot moist conditions. The large increase in dermal excretion of iodine under hot moist conditions overbalanced the urinary decrease, resulting in an increase under these conditions of 9.3  $\gamma$  in the combined hourly excretion of iodine. This was consistent for all three subjects.

Table IV gives the results obtained during 4 experimental weeks, during which the intake of iodine and the fecal excretion per day were determined in addition to the 24 hour urinary excretion and the 8 hour dermal excretion. There was very little difference between the dermal excretion of iodine under hot moist and comfortable conditions between Weeks 24 and 25. How-



TABLE II

*Loss of Iodine through Skin and Urine under Hot Moist and Comfortable Conditions*

Wk. No	Room conditions	Subject	Net loss in body weight	Iodine excretion			
				Dermal	Urinary	Combined	Urinary- dermal ratio
			gms per hr.	γ per hr	γ per hr.	γ per hr.	
13	Hot moist	B	402	4.7	14.1	18.8	3.0
		C	534	5.3	17.2	22.5	3.2
		D	753	5.5	20.9	26.4	3.8
		E	636	5.0	18.5	23.5	3.7
Average			581	5.1	17.7	22.8	3.5
14	Comfortable	B	65	2.4	19.7	22.1	8.2
		C	86	2.3	17.4	19.7	7.6
		D	118	2.0	20.3	22.3	10.2
		E	80	2.2	16.8	19.0	7.6
Average			87	2.2	18.6	20.8	8.5

TABLE III

*Loss of Iodine through Skin and Urine under Hot Moist and Comfortable Conditions with KI Dosage*

The supplement for both weeks was 2 gm of KI.

Wk. No	Room conditions	Subject	Net loss in body weight	Iodine excreted			
				Dermal	Urinary	Combined	Urinary- dermal ratio
			gm per hr	γ per hr.	γ per hr.	γ per hr.	
17	Hot moist	C	653	16.0	85.4	101.4	5.3
		D	879	23.3	93.5	116.8	4.0
		E	909	21.5	85.0	106.5	4.0
Average			814	20.3	88.0	108.3	4.3
18	Comfortable	C	101	1.1	94.1	95.2	85.5
		D	156	1.0	108.5	109.5	108.5
		E	82	1.1	91.2	92.3	82.9
Average			113	1.1	97.9	99.0	89.0

ever, with KI dosage under hot moist conditions the dermal excretion was about 7 times that under comfortable conditions.

The effect of dosage under both comfortable and hot moist conditions

is seen in a comparison of Week 25 with 28 and 24 with 27. Under comfortable conditions KI dosage resulted in an inappreciable increase of dermal excretion of iodine (from an average of 2.0 to 2.6  $\gamma$  per hour), but

TABLE IV

*Iodine Excretion under Hot Moist and Comfortable Conditions with and without KI Dosage*

Wk. No.	Room conditions	Supplement	Subject	Net loss in body weight	Iodine intake	Iodine excretion per hr.				Total iodine excretion		
						Dermal	Urinary	Fecal	Total	Dermal	Urinary	Fecal
				gm. per hr.	$\gamma$ per day	$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent	per cent	per cent
24	Hot moist	None	C	498	679	1.6	11.6	1.4	14.6	11.0	79.4	9.6
			D	738	819	0.8	15.4	1.9	18.1	4.4	85.1	10.5
			E	680	715	2.6	12.3	2.1	17.0	15.3	72.3	12.4
			F	438	595	1.0	8.8	1.4	11.2	8.9	78.6	12.5
			Average.....	589	702	1.5	12.0	1.7	15.2	9.9	78.9	11.2
25	Comfortable	None	C	86	629	2.2	22.3	2.4	26.9	8.2	82.9	8.9
			D	126	820	1.8	26.6	2.3	30.7	5.9	86.6	7.5
			E	66	716	1.9	22.3	1.3	25.5	7.5	87.4	5.1
			F	54	597	2.0	15.6	1.4	19.0	10.5	82.1	7.4
			Average.....	83	691	2.0	21.7	1.9	25.6	7.8	84.8	7.4
27	Hot moist	2 mg.* KI	C	635	2208	16.1	89.5	4.2	109.8	14.7	81.5	3.8
			D	863	2353	22.0	107.0	2.6	131.6	16.7	81.3	2.0
			E	969	2234	20.3	90.7	3.0	114.0	17.8	79.6	2.6
			F	410	2125	12.3	87.2	1.9	101.4	12.1	86.0	1.9
			Average.....	719	2230	17.7	93.6	2.9	114.2	15.5	82.0	2.5
28	Comfortable	2 mg.* KI	C	79	2208	2.5	80.0	1.2	83.7	3.0	95.6	1.4
			D	132	2352	2.7	103.5	1.5	107.7	2.5	96.1	1.4
			E	80	2246	2.5	86.5	1.4	90.4	2.8	95.7	1.5
			F	51	2123	2.5	80.0	1.2	83.7	3.0	95.6	1.4
			Average.....	86	2232	2.6	87.5	1.3	91.4	2.8	95.8	1.4

\* 2 mg. of KI are equivalent to 1528  $\gamma$  of iodine.

in a marked increase in urinary excretion of iodine (from an average of 21.7 to 87.5  $\gamma$  per hour). Under hot moist conditions with KI dosage there was an average increase in dermal excretion from 1.5 to 17.7  $\gamma$  per hour, and an increase in the urinary excretion from 12.0 to 93.6  $\gamma$  per hour.

## DISCUSSION

Whether the increase in dermal and urinary excretion of iodine on dosage with supplementary iodine is due merely to the increased iodine intake or is a reflection of a difference in the response of the body to dietary iodine from the response to supplementary KI is difficult to determine from our experiments. Davison and Curtis (8) found that all of the urinary iodine is acetone-soluble, which they presume is inorganic, regardless of the form of iodine ingested.

An important nutritional question with reference to iodine metabolism is the effect of a hot environment on the losses of iodine from the body and on iodine requirements, particularly as this effect may involve the activity of the sweat glands. Unfortunately the results of these experiments do not give unequivocal answers to these questions, even under such extreme environmental conditions as to induce net losses in body weight of about 92 gm. per hour in one environment and about 680 gm. per hour in the other environment. At the lower level of iodine intake (about 700  $\gamma$  daily), the dermal loss of iodine was but little, or not at all, affected by the differences in environment, while the urinary loss may actually be greater under non-sweating conditions, due possibly to a greater urine volume. At the higher level of iodine intake (about 2200  $\gamma$  daily), brought about by doses of KI, the dermal loss of iodine was markedly and significantly increased with variable and conflicting changes in urinary output. It would be hazardous to formulate a conclusion on the effect of environment on total iodine elimination from the body and on iodine requirements at either level of iodine consumption.

## SUMMARY

1. The concentration of iodine in undiluted sweat was 0.95  $\gamma$  per 100 cc. A single dose of 2 mg. of KI increased the average concentration to 3.18  $\gamma$  per 100 cc., while 14 daily doses of 2 mg. of KI did not produce any significant additional increase.

2. Profuse sweating (averaging about 677 gm. per hour) increases dermal losses of iodine at high levels of iodine intake (2200  $\gamma$  per day, KI dosage), but at low levels (700  $\gamma$  per day) no consistent or considerable effect was observed. The effect of a sweating environment on urinary output of iodine was too variable to permit a general statement. Thus, no evidence was obtained that a sweating environment will increase iodine requirements.

3. At least three-fourths of the total iodine lost from the body was excreted through the urine. When the iodine intake was increased with KI supplementation, most of the increased excretion of iodine was lost through the urine.

4. The fecal excretion of iodine was greater under hot moist than under comfortable conditions at high levels of iodine intake, but not at the basal levels. Iodine dosage raised the fecal output of iodine significantly in a hot moist, but not in a comfortable, environment.

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# THE EFFECT OF PANTOTHENIC ACID DOSAGE AND ENVIRONMENTAL TEMPERATURE AND HUMIDITY UPON THE DERMAL AND RENAL EXCRETION OF PANTOTHENIC ACID\*

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The loss of considerable amounts of water-soluble nutrients in the sweat would have an important bearing on nutrition in the tropics and in hot humid seasons of the year in the temperate zone, provided these losses represented something more than a mere diversion of excretion from kidney to sweat glands. The possibility of such losses has stimulated considerable research in recent years, particularly studies of the vitamin content of sweat.

The present paper, concerned with the manner of excretion of pantothenic acid by adult man on different levels of intake, is one of a series of papers on this general subject from this laboratory. Its significance in practical human nutrition cannot be assessed until the essentiality of pantothenic acid in the human diet has been more firmly established. The evidence on this point recently reviewed by Gordon (1) leaves much to be desired. In this connection it may be pointed out that the demonstration of a therapeutic value of a substance does not necessarily imply that a dietary deficiency has been thus corrected by it.

## EXPERIMENTAL

Five young men, 21 to 28 years of age, served as subjects of the experiment. For 8 hours each day, Monday through Friday of each experimental week, the subjects were kept in an air-conditioned room maintained at "comfortable" (about 28.9° and 50 per cent relative humidity) or "hot moist" (about 38.3° and 69 per cent relative humidity) conditions, the latter simulating a tropical (jungle) environment, and occasionally at other conditions. Dermal excretions were collected during the 8 hour periods in the experimental chamber and urinary excretions were collected for the entire 24 hours. During 8 of the experimental weeks these collections were preserved with toluene and analyzed for pantothenic acid. For 5 of the 8 weeks the subjects received a supplementary dose of pantothenic acid. On three Saturday mornings a 4 hour collection of undiluted

\* The data presented in this paper were taken from a project covered by a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

sweat was made under hot moist conditions for pantothenic acid analysis. The procedures for collecting complete dermal excretions and undiluted sweat are described in detail in a previous publication (2).

Before entering the exposure chamber, and immediately on leaving it, the men were weighed without clothing to the nearest gm. on a Troemner balance. The final body weight plus the weight of all urinary and fecal excreta subtracted from the initial body weight plus the weight of all food and water ingested gives the "net loss" in body weight. The net loss in body weight under comfortable conditions averaged 85 gm. per hour, representing largely insensible vapor loss. Under hot moist conditions, the net loss in body weight averaged 661 gm. per hour, representing mainly the secretion of the sweat glands.

Pantothenic acid was determined by a microbiological method with *Lactobacillus arabinosus* as the test organism and the improved basal medium of Krehl, Strong, and Elvehjem (3). Pantothenic acid is essential for *Lactobacillus arabinosus* (4), and the possibility of using this organism for the determination of pantothenic acid was first suggested by Snell and Wright (5) in 1941.

One of the reasons for selecting *Lactobacillus arabinosus* was that this organism was reported to be insensitive to fatty acids, when grown on the nicotinic acid basal medium of Krehl, Strong, and Elvehjem (3), whereas *L. casei* was reported (6, 7) to be stimulated by fatty products in the presence of suboptimal amounts of pantothenic acid. However, preliminary experiments showed that fat-soluble materials in sweat also stimulated acid production by *L. arabinosus* when sweat samples were assayed directly, yielding apparent pantothenic acid values approximately 3 times the values obtained after ether extraction or filtration through Filter-Cel, which completely eliminates this stimulatory effect. Since this work was completed, two procedures have been published (8, 9) which report the use of *L. arabinosus* in the microbiological determination of pantothenic acid. Both of these reports confirm the sensitivity of *L. arabinosus* to stimulation by fatty materials.

Enzyme (clarase) treatment did not yield higher values, indicating that all the pantothenic acid in sweat is present in the free form. All samples were filtered through Filter-Cel and assayed directly. The pantothenic acid values are expressed in terms of calcium pantothenate.

### Results

The concentration of pantothenic acid in undiluted sweat is given in Table I. No increase with dosage is indicated and the total average was 3.8  $\gamma$  per 100 cc. with a standard error of the mean of  $\pm 0.56$ .

Table II shows the effect upon the dermal excretion of pantothenic acid

of an increase in humidity with approximately the same dry bulb temperature. A dose of 8 mg. of calcium pantothenate was given daily. When the relative humidity in the chamber was raised from 65 to 92 per cent, there was a 176 per cent increase in the average net loss in body weight

TABLE I

*Pantothenic Acid Concentration in Undiluted Sweat with and without Dosage*

The results are given in micrograms per 100 cc.

Wk. No. (Saturday)	Pantothenic acid supplement before sweat collection	Subject C	Subject D	Subject E	Subject F	Group average
26	None	3.7	5.0	3.9	7.7	5.1
38	"	1.6	5.4	5.0	1.5	3.4
39	7 daily doses of 16 mg. calcium pantothenate*	1.2	3.0	4.4	3.2	3.0
Individual average.....		2.2	4.5	4.4	4.1	3.8

\* Plus four 1 gm. tablets of Mead's brewers' yeast.

TABLE II

*Effect of Humidity upon Dermal Excretion of Pantothenic Acid*

8 mg. of calcium pantothenate were given daily.

Wk.No.	Chamber, dry bulb	Conditions, relative humidity	Subject	Net loss in body weight	Excretion of pantothenic acid	
	°C.	per cent		gm. per hr.	γ per hr.	γ per 100 gm. weight loss
3	32.22	65	B	122	1.7	1.4
			C	138	1.8	1.3
			D	221	8.7	4.0
			E	140	2.8	2.0
Average ..				155	3.8	2.2
7	32.77	92	B	155	7.9	5.1
			C	369	7.9	2.1
			D	626	18.6	3.0
			E	562	14.5	2.6
Average.....				428	12.2	3.2

with a 221 per cent increase in the average dermal excretion of pantothenic acid. No significant increase in the concentration of pantothenic acid in sweat is indicated by the ratio of the dermal loss of the vitamin to the net loss in body weight under environmental conditions differing only in humidity.



A comparison of the hourly loss of pantothenic acid through the skin and through the kidneys under hot moist conditions with that under comfortable conditions is presented in Table III. In Weeks 21 and 22, when the diet was supplemented daily with 8 mg. of calcium pantothenate,

TABLE III  
*Dermal and Urinary Excretion of Pantothenic Acid under Hot Moist and Comfortable Conditions*

Wk. No.	Room conditions	Calcium panto- thencate supple- ment	Subject	Net loss in body weight	Pantothenic acid excretion				Combined loss through skin	Volume of urine per 24 hrs.
					Dermal		Urinary	Com- bined		
		mg.		gm. per hr.	$\gamma$ per hr.	$\gamma$ per 100 gm. weight loss	$\gamma$ per hr.	$\gamma$ per hr.	per cent	cc.
21	Hot moist	8	C	477	6.5	1.4	379	386	1.7	884
			D	697	19.0	2.7	431	450	4.2	1008
			E	771	9.7	1.3	381	391	2.5	984
			F	377	7.2	1.9	293	300	2.4	1152
Average .....				581	10.6	1.8	371	382	2.7	1007
22	Comfortable	8	C	81	7.6	9.4	303	310	2.4	1730
			D	127	8.5	6.7	318	326	2.6	1860
			E	68	5.8	8.5	329	335	1.7	2322
			F	59	5.4	9.2	296	301	1.8	1970
Average .....				84	6.8	8.5	311	318	2.1	1971
27	Hot moist	None	C	635	20.4	3.2	252	272	7.5	672
			D	863	38.3	4.4	293	332	11.6	782
			E	969	34.4	3.6	200	235	14.7	728
			F	410	17.7	4.3	87	104	17.0	945
Average .....				719	27.7	3.9	208	236	12.7	782
28	Comfortable	None	C	79	7.7	9.7	228	235	3.3	2482
			D	132	4.3	3.3	238	242	1.8	2478
			E	80	3.5	4.4	176	180	1.9	2640
			F	51	4.9	9.6	183	188	2.6	3030
Average .....				86	5.1	6.8	206	211	2.4	2658

three of the four subjects showed an increased dermal excretion of pantothenic acid under hot moist conditions. It is of interest to observe that there was also an increased urinary excretion of pantothenic acid under hot moist conditions for three of the four subjects. This increase is

especially significant since the average volume of urine was 1971 cc. under comfortable conditions and only 1007 cc. under hot moist conditions.

On a constant diet without dosage in Weeks 27 and 28 all subjects showed a marked increase in dermal excretion of pantothenic acid under hot moist conditions. The average increase was 443 per cent. With the exception of one subject (F) all showed also an increase in the urinary excretion of pantothenic acid. This subject (F) also showed no significant change in the urinary excretion of pantothenic acid in Weeks 21 and 22. The average

TABLE IV  
*Effect of Dosage upon Dermal and Urinary Excretion of Pantothenic Acid under Hot Moist Conditions*

Wk No.	Room conditions	Pantothenic acid supplement	Subject	Net loss in body weight	Pantothenic acid excretion				Combined loss through skin
					Dermal		Urinary	Combined	
		mg.		gm. per hr.	$\gamma$ per hr.	$\gamma$ per 100 gm weight loss	$\gamma$ per hr.	$\gamma$ per hr.	per cent
38	Hot moist	None	C	594	23.7	4.0	156	180	13.2
			D	693	24.5	3.5	162	186	13.2
			E	736	30.8	4.2	91	122	25.3
			F	664	21.5	3.2	132	153	14.0
			Average . . .	672	25.1	3.7	135	160	16.4
39	Hot moist	16*	C	588	21.3	3.6	346	367	5.8
			D	632	23.9	3.8	420	444	5.4
			E	776	30.8	4.0	405	436	7.1
			F	687	25.0	3.6	298	323	7.7
			Average . .	671	25.3	3.8	367	393	6.5

\* Plus twelve 1 gm. tablets of Mead's brewers' yeast.

volume of urine was 2658 cc. under comfortable conditions and only 782 cc. under hot moist conditions.

The increase in dermal excretion of pantothenic acid under hot moist conditions was so much greater than the increase in urinary excretion that the per cent of the total excretion of pantothenic acid lost through the skin rose from 2.4 under comfortable conditions to 12.7 under hot moist conditions.

Table IV shows the effect of dosage upon the dermal and renal excretion of pantothenic acid under hot moist conditions. There was no significant change in the concentration of pantothenic acid in sweat with the dosage given, as indicated by the value of pantothenic acid per 100 gm. net loss

in body weight per hour. However, this dosage produced an increase of 172 per cent in the urinary excretion of pantothenic acid.

#### DISCUSSION

The concentration of pantothenic acid in sweat found in these studies (average, 3.8  $\gamma$  per 100 cc.) is considerably lower than that reported by Tennent and Silber (10) (2 to 30  $\gamma$  per 100 cc.), or by Cornbleet *et al.* (11) (12 to 80  $\gamma$  per 100 cc.). The higher concentration found by these investigators can probably be attributed to the stimulating effect of fatty materials in sweat upon the test organism, *Lactobacillus casei*, used in the determination of pantothenic acid.

Dosage with about 18 mg. of pantothenic acid did not increase the concentration of this vitamin in undiluted sweat nor its hourly excretion through the skin under hot moist conditions. This is contrary to the report of Tennent and Silber (10) who stated that the average excretion of pantothenic acid in sweat was 24  $\gamma$  per hour for subjects who were not dosed and 50  $\gamma$  per hour for those who received a supplement of 50 mg. of calcium pantothenate daily. Examination of their data reveals that in only two cases were the same subjects tested with and without dosage: subject R. S. excreted 15  $\gamma$  per hour without and 20  $\gamma$  per hour with dosage, and subject J. F. excreted 20  $\gamma$  without and 21  $\gamma$  per hour with dosage. In both cases the sweat was induced by exercise.

An increase in the relative humidity resulted in an increase in net loss of body weight, representing mainly the secretion of the sweat glands, and generally a concomitant increase in the dermal excretion of pantothenic acid. When the difference between the average net loss in body weight under hot moist conditions and that under comfortable conditions is multiplied by the average concentration (3.8  $\gamma$  per 100 cc.) of pantothenic acid in sweat, the values obtained closely approximate the actual excretion found.

The ratio of the dermal loss of pantothenic acid to the net loss in body weight under hot moist conditions reveals, in three out of four cases, the same average concentration of the vitamin in sweat as was obtained by actual determination on undiluted sweat. In the 2 comfortable weeks this ratio indicates that the insensible dermal water loss has a higher concentration of pantothenic acid than that of sweat.

Under hot moist conditions (Week 39) 31 per cent of an 18 mg. dose of pantothenic acid was recovered in the urine. This is higher than the 2.8 to 11.7 per cent found by Pearson (12) for a dose of 1 gm. The average daily urinary excretion of pantothenic acid on a diet without dosage was 3.24 mg. in 1 week and 4.99 mg. in another. This is in agreement with the reports in the literature: Pelczar and Porter (13), average 3.81 mg.;

Pearson (12), 3.12 to 3.3 mg.; Wright and Wright (14), average 3.42 mg.; and Gordon (1), 3.52 mg.

The combined urinary and dermal excretion of pantothenic acid under hot moist conditions on an average diet was 5.657 mg. per day in Week 27 and 3.842 mg. per day in Week 38.

Comparison of the results of Week 28 with 27 and of Week 22 with 21, with reference to change in combined hourly excretion of pantothenic acid, by the *t* test of "Student" (15) gives a *P* value of 0.056. This comparison suggests strongly, but does not demonstrate, that environmental conditions inducing profuse sweating increase the total output of pantothenic acid from the body.

#### SUMMARY

Dermal excretions for a period of 8 hours and urines for 24 hours were collected from five young men under comfortable and under hot moist conditions, both with and without pantothenic acid dosage. These collections were analyzed for pantothenic acid by a microbiological method with *Lactobacillus arabinosus* as the test organism.

1. Dosage with about 18 mg. of pantothenic acid daily did not affect the pantothenic acid concentration of undiluted sweat, which averaged 3.8  $\gamma$  per 100 cc. The insensible dermal water loss in the comfortable conditions showed a higher concentration of pantothenic acid than sweat.

2. An increase in the relative humidity from 65 to 92 per cent with a dry bulb temperature of about 32.22° produced an increase of 176 per cent in the average net loss of body weight, representing mainly the secretion of the sweat glands, and a concomitant increase of 221 per cent in the average dermal excretion of pantothenic acid.

3. On an unsupplemented diet the average dermal excretion of pantothenic acid was 5.1  $\gamma$  per hour under comfortable conditions. Under hot moist conditions the dermal excretion of pantothenic acid increased to 27.7  $\gamma$  per hour. An increase in the urinary excretion of pantothenic acid was also observed under hot moist conditions, even though the volume of urine under these conditions was only about one-half to one-third of the volume under comfortable conditions. Under hot moist conditions 31 per cent of an 18 mg. dose of pantothenic acid was recovered in the urine.

4. The combined urinary and dermal excretion of pantothenic acid under hot moist conditions on a normal diet averaged 5.66 mg. per day in 1 week and 3.84 mg. per day in another. This is an increase of 11.6 per cent over the excretion under comfortable conditions. The data suggest, but do not prove, that the total excretion of pantothenic acid in urine and sweat may be increased under conditions of profuse sweating induced by environmental conditions.

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## OXYGEN POISONING

### V. THE EFFECT OF HIGH OXYGEN PRESSURE UPON ENZYMES: SUCCINIC DEHYDROGENASE AND CYTOCHROME OXIDASE\*

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Since its first proposal by Bert (1), the hypothesis that oxidative enzymatic systems in animals are inhibited by high pressures of oxygen has occupied a prominent place in discussions of the phenomenon of oxygen poisoning (2, 3). In particular the succinoxidase system (succinic dehydrogenase and cytochrome oxidase) has been considered by some as one whose inactivation might explain the whole train of ill events which follow the exposure of animals to excessive pressures of oxygen.

Inhibiting action of oxygen upon succinic dehydrogenase of tissues has been reported by Lehman (4) and Bean and Bohr (5). Libbrecht and Massart (6) gave to cytochrome oxidase a central rôle: at high pressures oxygen reacts with it to form *oxygène actif* which in turn inactivates succinic dehydrogenase. They concluded that molecular oxygen *per se* has no toxic action, because they found no inhibition of succinic dehydrogenase by oxygen at high pressures when the cytochrome oxidase present in their preparations was inhibited by cyanide.

In this paper we report further experiments on inactivation of the succinoxidase system of tissues from rats at high oxygen pressures. Detailed consideration is given to the hypothesis of Libbrecht and Massart. The mechanism of inactivation by high oxygen is considered and experiments are reported bearing on it.

#### EXPERIMENTAL

*Tissue Preparations*—These were made from white rats. Homogenates were prepared by the Potter-Elvehjem method (7). Slices were 0.35 or 0.5 mm. thick and prepared as described elsewhere (8). Unusual details of preparations are described under the individual experiments.

*Determination of Succinoxidase Activity*—Two methods were used, aerobic and anaerobic.

*Aerobic Method*—10 to 50 mg. (wet weight) of homogenized tissue were

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

placed in the main compartment of a Warburg vessel with 3.0 ml. of 0.1 M phosphate (pH 7.4), 0.004 M  $\text{CaCl}_2$ , 0.004 M  $\text{AlCl}_3$ , and optimal amounts of cytochrome c. The side sac contained 0.1 ml. of 1 M sodium succinate and an alkali filter paper roll inset was placed in the center well. The vessels were gassed with 100 per cent oxygen and after temperature equilibration at 38° the succinate was admixed with the medium. The oxygen uptake was then measured over a 30 to 40 minute period, during which time it was rectilinear ( $\pm 5$  per cent). The activity was expressed as micromoles of succinate oxidized per gm. of wet tissue per hour, 2 moles of succinate being equivalent to 1 mole of oxygen. In some instances several amounts of tissue were assayed and the activity calculated from the slope of the line through the points representing oxygen uptake *versus* tissue weight.

*Anaerobic Method*—This was essentially that described by Quastel and Wheatley (9). The tissue was suspended in a bicarbonate medium, ferricyanide and succinate being in the side sac. The gas phase was nitrogen with 5 per cent  $\text{CO}_2$ . Upon admixture the succinate is oxidized by the ferricyanide, *viz.*  $\text{Fe}(\text{CN})_6^{=+} + \text{H} = \text{Fe}(\text{CN})_6^{\equiv} + \text{H}^+$ . The hydrogen ion reacts with bicarbonate and the evolved  $\text{CO}_2$  is measured manometrically. Moles of succinate oxidized were calculated as half the moles of  $\text{CO}_2$  formed. The medium had the following composition: sodium bicarbonate 0.040 M, sodium succinate 0.12 M,  $\text{AlCl}_3$  and  $\text{CaCl}_2$  0.0005 M. 100 micromoles of ferricyanide appeared to be the optimal amount to be added from the side sac.

The following remarks sum up our experience with the method: (1) The use of yellow phosphorus in the center well, recommended by Quastel and Wheatley, to remove traces of oxygen appears unnecessary and was omitted in most of our experiments. (2) Upon admixture of the ferricyanide and medium there is a momentary rapid evolution of  $\text{CO}_2$ . This is an artifact and is ignored. After 1 to 2 minutes there is a steady  $\text{CO}_2$  evolution which is constant in rate for 20 to 30 minutes. The activity is calculated from this. (3) In the absence of tissue,  $\text{CO}_2$  evolution is negligible. (4) The activity is proportional to the amount of tissue, provided this is not too large. When several amounts of tissue are used, a better evaluation of activity can be calculated from the activity-tissue weight line. (5) This line usually cuts the abscissa at 0 to 5 mg., indicating, perhaps, an absorption of inactivating impurities in the system by an amount of tissue. (6) Amounts of ferricyanide and succinate equivalent to about 10 times the amount to be oxidized are approximately optimal. (7) The bicarbonate can be varied from 0.020 to 0.050 M with no significant difference in activity. This corresponds to a pH range of from 7.3 to 7.7. (8) Addition of calcium and aluminum ions increases the oxidation 20 to 30 per cent when the activity is calculated from that of one amount of tissue. There is little

effect if several amounts of tissue are used and the activity is calculated from the slope of the activity-tissue weight line. (9) As a rule the anaerobic and aerobic methods give the same activity for a given tissue. However, in the absence of added cytochrome, the aerobic method gives lower results. Our experience gave us confidence in the Quastel-Wheatley method for determination of succinic acid dehydrogenase. It is technically easy and has the advantage that no cytochrome is required. However, we have also used the aerobic method in suitable experimental studies of the inactivation effects of high oxygen pressure.

*Determination of Cytochrome Oxidase Activity. Oxygen Uptake in Presence of p-Phenylenediamine*—Either slices of tissue or homogenates were used. The medium containing 0.040 M phosphate (pH 7.3), 0.080 M NaCl, 0.005 M KCl, 0.002 M MgCl<sub>2</sub>, and 0.001 M CaCl<sub>2</sub>. Determinations were done in vessels of the Warburg type. The tissue being in the main

TABLE I

*Succinoridase Activity of Rat Tissue As Homogenate Determined Aerobically and Anaerobically (38°)*

Tissue	Succinate oxidized, micromoles per gm. per hr.	
	Aerobic	Anaerobic
Liver . . . . .	1160	1000
" . . . . .	1140	1110*
Kidney . . . . .	940*†	1620*

\* From the slope of the activity-tissue weight line, with three different amounts of tissue.

† No added cytochrome c present.

vessel, 0.2 ml. of 0.2 M *p*-phenylenediamine was tipped in from the side sac after temperature equilibration. The oxidase activity was expressed as micromoles of oxygen per gm. of wet tissue per hour, calculated from the readings of the first 20 minutes. 100 mg. of tissue as homogenate or slice were the customary amount used in each determination. In all cases parallel samples were set up, one without tissue and the other without *p*-phenylenediamine. Appropriate corrections were made from these controls in calculating the cytochrome oxidase activity of the samples.

*Oxygen Uptake in Presence of Ascorbic Acid and Cytochrome c*—The ability of the tissues to catalyze the oxidation of ascorbic acid in the presence of cytochrome *c*, as described by Schneider and Potter (10), was used to determine cytochrome oxidase activity. The medium in the main vessel of the Warburg respirator consisted of 0.25 ml. of 0.4 M sodium phosphate (pH 7.4), 0.3 ml. of 0.004 M AlCl<sub>3</sub>, 0.2 micromole of cytochrome *c*, and



water to 3.0 ml. Amounts of tissue as homogenates were added to give convenient activity. The side sac contained 0.3 ml. of neutralized ascorbic acid. An alkali filter roll inset was included. The gas phase was air. After temperature equilibration the contents of the side sac and main compartment were admixed, and the rate of oxygen uptake measured for 20 to 30 minutes. Two amounts of tissue were used and the activity expressed as micromoles of oxygen uptake per gm. of wet tissue per hour.

*Cytochrome c*—Two preparations were used, one from pigeon breast muscle by the method of Potter and DuBois (11), the other from horse heart by the method of Keilin and Hartree (12). The final solutions were dialyzed

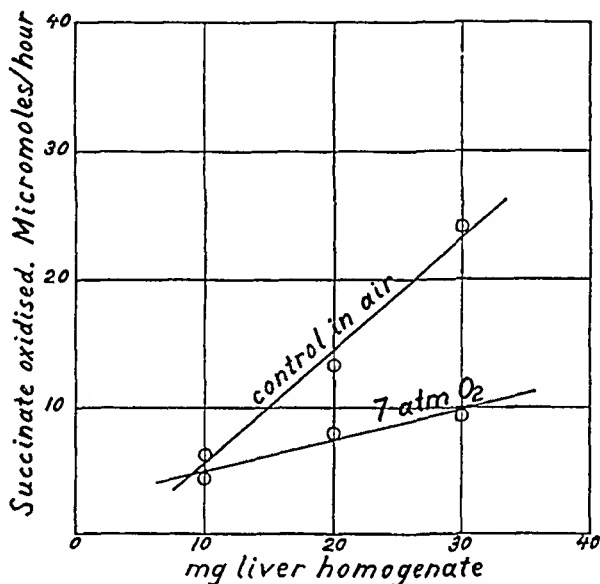


FIG. 1. Succinic dehydrogenase activity of rat liver homogenate after preliminary exposure at 38° for 2 hours to air or 7 atmospheres of oxygen; anaerobic assay.

against running water for 2 hours. The cytochrome concentration of the second preparation was determined spectrophotometrically.

*Succinic Dehydrogenase Activity of Tissue Homogenates after Exposure to High Oxygen Pressure*—Homogenates prepared from rat liver were exposed to 7 atmospheres of oxygen at 38° for 2 hours, subsequent to which the succinoxidase activity was determined by the anaerobic method with three different amounts of tissue. For comparison a control sample of the tissue was maintained in air at 38°. The data of a representative experiment (Fig. 1) show that compared to activity of the control rate of 900 micromoles per gm. per hour the sample exposed to high oxygen has been approximately 70 per cent inactivated in 2 hours.

Rat liver homogenate was exposed to 7 atmospheres of oxygen for varying periods of time and then assayed for succinoxidase activity by the anaerobic method. The results shown in Fig. 2 were obtained. The inactivation is relatively slow, 2 to 2.5 hours being required before half the activity is lost compared to control samples maintained in air at the same temperature. The same results were obtained if the activity was measured by the aerobic method. In general, the inactivation is slight during the 1st hour at

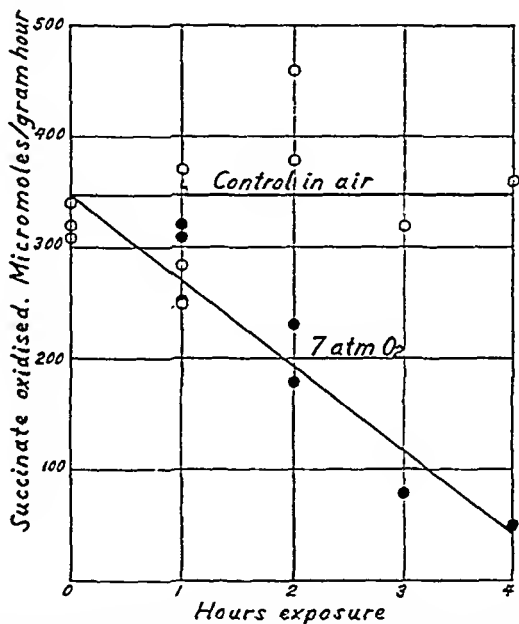


FIG. 2. Succinic dehydrogenase activity of rat liver homogenate after preliminary exposure at 38° for various periods of time to air or 7 atmospheres of oxygen; anaerobic assay.

approximately 7 atmospheres. However, there is considerable variation and occasionally considerable early inactivation was found. For example in one case tested by the aerobic method, the activity of a liver homogenate fell from 1400 to 700 micromoles per gm. per hour following 1 hour's exposure to 7 atmospheres of oxygen.

*Tissue Slices after Exposure to High Oxygen Pressure*—Control experiments showed that different weighed slices from the same liver did not differ significantly from each other when homogenized in saline or water and then

assayed for succinic dehydrogenase activity by the anaerobic method. Furthermore, slices of liver suspended in saline and equilibrated at 38° in air up to 4 hours, and then assayed as homogenates by the anaerobic method, were found to have the same activity as fresh slices so assayed. Accordingly it was possible to measure the effect of high oxygen pressures upon the succinoxidase activity of slices. Following preliminary exposure, the slices were homogenized and the activity determined with two or three different amounts of tissue. Table II shows the results of such experiments. In general, the enzyme in the slice was about as susceptible to the action of oxygen as it was in the homogenate. This is in contrast to other enzyme systems (*e.g.*, *d*-amino acid oxidase) which appear to be completely protected from the inactivating effect of oxygen when in the intact cell.

TABLE II

*Effect of 7 Atmospheres of Oxygen on Succinic Dehydrogenase Activity of Rat Liver Slices*

Rat No.	Preliminary period	Subsequent activity		
		Succinate oxidized, micromoles per gm. per hr.		Per cent of control
		Air	7 atmospheres O <sub>2</sub>	
	<i>hrs.</i>			
1	2	850	730	86
	4	710	480	68
2	2	914	1152	126
	4	922	613	66
3	2	585	260	44
	4	930	133	14

The succinic dehydrogenase activities were determined by the anaerobic assay. Two amounts of tissue were used for each determination and the activity was obtained from the slope of the activity-tissue weight line.

*Rat Kidney Homogenate*—When exposed to high pressures of oxygen and assayed in the same way as liver homogenate, it was found that this tissue appears to be quite susceptible to oxygen. At 38° the control samples had to be maintained under nitrogen, since relatively rapid inactivation was observed with oxygen at 1 atmosphere. Representative data are shown in Fig. 3. Within 4 hours at this pressure the succinoxidase activity was reduced to about one-third of the initial value.

*Mechanism of Succinoxidase Inactivation by High Oxygen*—Libbrecht and Massart (6) reported that succinoxidase preparations from liver or muscle to which cyanide had been added to inactivate the cytochrome oxidase system were unaffected by high oxygen pressures, in contrast to controls without cyanide. The activity of the enzyme system was deter-

mined aerobically at 8 atmospheres of oxygen, methylene blue being used to replace the cytochrome system inhibited by cyanide. On the basis of these experiments they formulated the following hypothesis of the mechanism of oxygen poisoning: Oxygen at high pressures reacts with cytochrome oxidase to produce *oxygène actif* which is toxic and inactivates succinic acid dehydrogenase. Molecular oxygen itself is not toxic; hence there is no inactivation when the cytochrome oxidase system is inactivated by cyanide. The precise meaning of the term *oxygène actif* is obscure, but it was interpreted by the present authors (2) to mean that the cytochrome oxidase

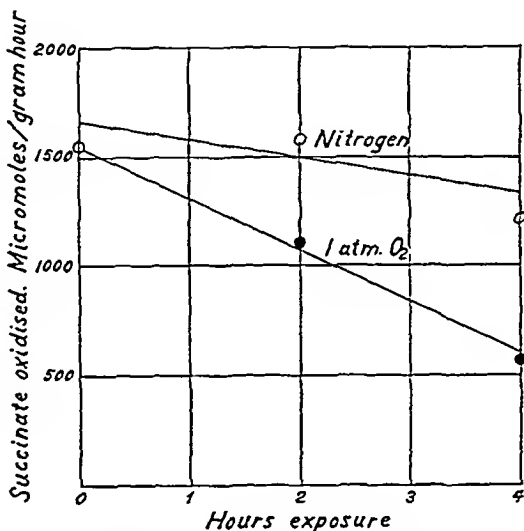


FIG. 3. Succinic dehydrogenase activity of rat kidney homogenate after preliminary exposure at 35° to nitrogen or 1 atmosphere of oxygen for various periods of time; anaerobic assay.

system under high oxygen pressure oxidized the succinic dehydrogenase to an inactive form.

This hypothesis has been advanced by Bean and Bohr (13) as one which could explain their experiments on muscle tonus. They found that the isolated pyloric sphincter of rabbits when subject to 5 to 6 atmospheres of oxygen rapidly lost its tonus, but that this effect could be prevented if cyanide was present in the medium in which the muscle was suspended.

In a series of five experiments, duplicate samples of liver homogenate prepared from rat liver and suspended in a phosphate buffer were exposed

to 7 atmospheres of oxygen for 1 hour. One sample contained KCN at concentrations varying from 0.25 to 2.5 mM per liter. Subsequent to the preliminary exposure to high oxygen pressure, the samples were assayed for succinoxidase activity in the presence of added succinate. Since the cytochrome oxidase system had been inactivated, methylene blue was added in optimal concentration to act as hydrogen acceptor. During the assay period both samples were identical with respect to cyanide, succinate, and methylene blue. In no case was there any indication that the presence of cyanide during the period of exposure to high oxygen had prevented the inactivation of the succinoxidase activity.

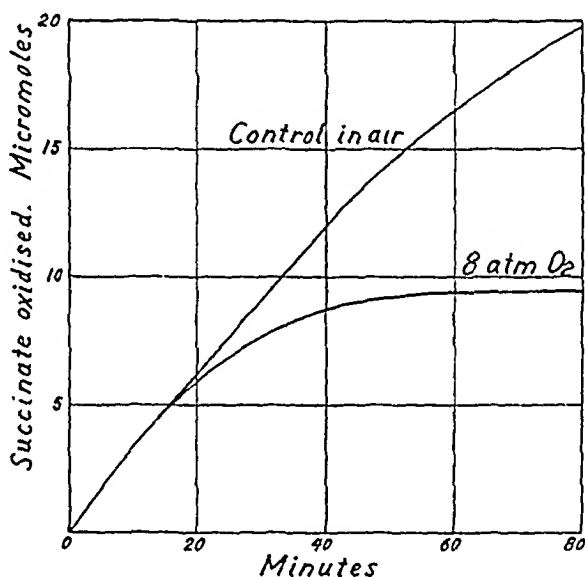


FIG. 4. Succinate oxidation of a pigeon breast muscle extract in the presence of 0.010 M KCN and 0.002 M methylene blue in air or 8 atmospheres of oxygen; 38°.

In a second experiment an enzyme preparation was made from pigeon breast muscle by extraction with phosphate (pH 7.4). The activity of this preparation was measured as the rate of oxygen uptake in the presence of succinate (38°). Control experiments showed that this preparation was completely inactive in the presence of 0.010 M KCN. The further addition of methylene blue to 0.002 M, however, restored the activity to about 60 per cent of the initial level. The activities of two samples of this preparation were measured as follows: (1) in the air with 0.002 M methylene blue and 0.010 M KCN, and (2) in oxygen at 8 atmospheres with the same concentrations of methylene blue and cyanide. The results, presented in

Fig. 4, show that the sample at high oxygen pressure was rapidly inactivated in agreement with our experience in the absence of cyanide. The sample assayed in air showed only a slight decrease of activity. In other words, there is no indication that the presence of cyanide prevents the inactivation of succinic dehydrogenase by oxygen. On the contrary, inactivation was more rapid than in its absence.

In a third experiment, similar in character, a homogenate was prepared from rat brain and the activities of three samples were measured aerobically in the presence of added succinate (0.020 M). The data are shown in Fig. 5.

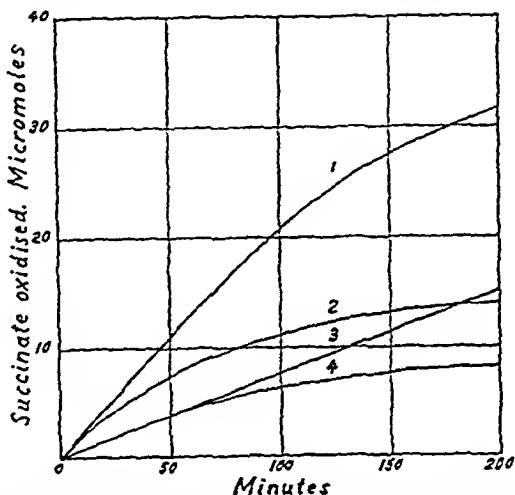


FIG. 5. Succinate oxidation at 38° by rat brain homogenate: Curve 1, in air + 0.002 M methylene blue; Curve 2, 7 atmospheres of oxygen + 0.002 M methylene blue; Curve 3, air + 0.002 M methylene blue and 0.010 M KCN; Curve 4, 7 atmospheres of oxygen + 0.002 M methylene blue and 0.010 M KCN.

The following may be noted. A sample with methylene blue (0.002 M) continued its activity in air for a period of 200 minutes, the rate falling off only slowly (Curve 1). A similar sample in oxygen at 7.2 atmospheres showed the rapid loss of activity characteristic of oxygen poisoning (Curve 2). A third sample containing cyanide (0.010 M) in addition to methylene blue had a linear oxygen uptake in air for a period of 200 minutes but showed a loss in activity under high pressure of oxygen (Curve 3).

Libbrecht and Massart stated that aged succinoxidase preparations lose the capacity to oxidize succinate aerobically unless methylene blue is present. Presumably the cytochrome oxidase system becomes inactive,

whereas the succinic dehydrogenase system retains its activity. Such a system according to these authors is not inactivated by high oxygen pressure, a finding in conformity with their hypothesis that no *oxygène actif* will be formed if cytochrome oxidase is not in the system. We made many attempts by aging and heating at moderate temperatures to prepare a succinoxidase system from pigeon breast muscle extract which was active with methylene blue and *completely* inactive without. In this we were unsuccessful. However, we succeeded in obtaining a preparation whose activity in oxidizing succinate in air was more than doubled by the addition of methylene blue. Experiments with this preparation (Fig. 6) showed

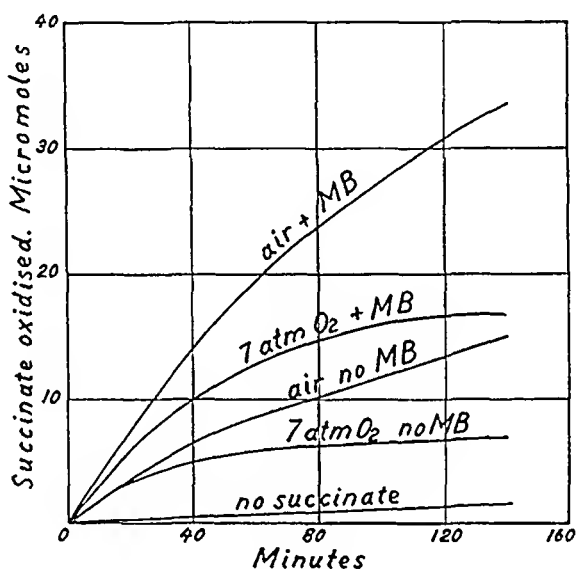


FIG. 6. Aged pigeon breast muscle extract deficient in cytochrome oxidase; succinate oxidation in air and 7 atmospheres of oxygen with and without methylene blue.

that in air (methylene blue present) the activity continued relatively unaltered for 80 to 100 minutes; in 7.2 atmospheres of oxygen there was rapid inactivation; in the absence of methylene blue the inactivation was still more rapid. There is no indication in these experiments with a system relatively poor in cytochrome oxidase, so that only half or less of the total oxygen is carried by it, that the succinic acid dehydrogenase is less susceptible to high oxygen pressures.

A fifth type of experiment was done in which the succinic acid dehydrogenase activity was measured anaerobically by the method already described. Homogenates of rat liver in phosphate (pH 7.4) were equilibrated at 38° for 1 hour at 6.9 atmospheres of oxygen. The preparations were

then assayed for activity anaerobically. The data are given in Table III from which it may be noted that (1) the preparation was inactivated 50 per cent in 1 hour at 6.9 atmospheres of oxygen; (2) KCN at 0.001 M (sufficient to inhibit completely the cytochrome oxidase as shown by preliminary aerobic experiments) did not prevent the inactivation, nor did KCN up to 0.010 M; (3) the oxygen inactivation tended to be greater the higher the KCN concentration, although the KCN has little effect on the anaerobic activity as determined by controls.

*Relation of Sulfhydryl Groups to Oxygen Poisoning*—It was shown by Hopkins and Morgan (14) that succinic dehydrogenase is dependent for its activity on the presence in the enzyme molecule of sulfhydryl groups. When these are oxidized to the thiol state, the enzyme is inactivated. Upon reduction back to the sulfhydryl form, the enzyme is reactivated. These observations have been confirmed, notably by Potter and DuBois

TABLE III

*Inactivation of Succinic Dehydrogenase by High Oxygen Pressure in Presence and Absence of Cyanide*

Rat liver homogenate in 7.4 phosphate at 38°; anaerobic assay method.

Preliminary period, 1 hr.		Subsequent activity	
$pO_2$	KCN	Succinate oxidized	Per cent of control
atmospheres	millimoles per l.	micromoles per gr. per hr.	
0.2	0	245	
6.9	0	123	50
6.9	1	93	38
6.9	5	40	16
6.9	10	24	10

(15) who, upon the basis of their experiments, have advanced a theory explaining succinate oxidation.

Our own experiments are in complete conformity with these conceptions. For example when *o*-iodosobenzoate is used as the oxidizing agent, the results shown in Table IV were obtained.

Rat diaphragm or slices of liver or heart are, in most instances, markedly inhibited in their succinic acid dehydrogenase activity by the prior addition of small amounts of *o*-iodosobenzoate. In Experiment 6 a homogenate of rat liver was prepared from washed liver slices. The amount of *o*-iodosobenzoate necessary to oxidize all free —SH groups was determined by the method described by Hellerman, Chinard, and Ramsdell (16). This consists in adding an excess of the oxidizing agent by an iodometric titration. The method gives a fairly good estimate of the total free —SH groups in the homogenate. In this case the addition of *o*-iodosobenzoate



equivalent to 125 per cent of the total calculated free —SH groups resulted in practically complete inhibition of the succinic acid dehydrogenase activity.

These considerations at once raise the question, is the action of oxygen at high pressures that of an oxidizing agent to oxidize the free sulfhydryl groups of succinic dehydrogenase and thus inactivate it? Our further experiments tend to answer this question affirmatively.

TABLE IV

*Inactivation of Succinic Dehydrogenase of Rat Tissues by 30 Minutes Preliminary Treatment with o-Iodosobenzoate*

Bicarbonate-saline medium; 38°; anaerobic method of assay.

Experiment No.	Weight of tissue	Total o-iodosobenzoate added	Succinate oxidized	Inhibition
	mg.	micromoles	micromoles per gm. per hr.	per cent
1. Liver slice	49	0.0	248	
	39	1.0	84	66
	45	5.0	19	92
2. Heart "	46	0.0	154	
	56	1.0	28	82
	82	5.0	18	88
3. Diaphragm	119	0.0	94	
	135	10.0	17	82
4. Heart slice	78	0.0	104	
	73	0.010	128	0
	73	0.10	96	8
	87	1.0	42	60
5. Diaphragm	61	0.0	196	
	73	0.01	110	±0
	61	0.10	116	±0
	69	1.0	68	36
6. Liver homogenate	30	0.0	730	
	30	2.7*	17	98

\* Equivalent to 125 per cent of total estimated free —SH groups.

For example, it has been shown repeatedly (Hopkins and Morgan (14); Potter and DuBois (15)) that reduced glutathione or cysteine will reduce and thereby reactivate succinic acid dehydrogenase previously inactivated by oxidation. In Table V are given data showing that succinic dehydrogenase inactivated by preliminary treatment with high oxygen pressure can be similarly reactivated by treatment with reduced glutathione. A rat liver homogenate (100 mg. per ml.) was prepared in distilled water, divided into aliquots, and treated for a preliminary period of 2 hours as indicated. A second period followed in which one aliquot was treated with

reduced glutathione. Following this the samples were assayed by the anaerobic method. In each case two amounts of tissue were used and the activity calculated from the activity-tissue weight line. The presence of reduced glutathione during the assay period introduces no error, for we have repeatedly found that at the beginning of the assay period following the admixture of ferricyanide to the tissue plus succinate any glutathione present is rapidly and completely oxidized within 5 minutes. Thereafter evolution of  $\text{CO}_2$  is entirely due to oxidation of succinate. Consideration of Table V shows that succinic dehydrogenase was inactivated by high oxygen pressure, but subsequent treatment with reduced glutathione

TABLE V

*Reactivation by Reduced Glutathione of Succinic Dehydrogenase of Rat Liver Homogenates Preliminarily Inactivated by Oxygen at 7 Atmospheres*

Preliminary period, 2 hrs., 38°		Reactivation period, 2 hrs., 38°	Subsequent dehydrogenase activity			
Gas	Pressure	Reduced glutathione	Tissue	$\text{CO}_2$ evolved	Succinate oxidized*	Per cent of control
	atmospheres	micromoles per ml.	mg.	micromoles per 20 min.	micromoles per gm per hr.	
$\text{O}_2$	7	0	20	0.8		
"	7	0	40	2.2	105	26
"	7	36	20	2.3		
"	7		40	6.0	277	69
$\text{N}_2$	1	0	20	5.5		
		0		10.8	400	100†
"	1	36	20	6.9		
		36	40	11.6	353	88
Original hamagenate			20	6.9		
			40	13.5	495	125

\* Calculated from the slope of the two independent determinations.

† Control sample.

increased the activity 2.5-fold. Two controls in nitrogen with and without an intermediate period with glutathione were not significantly different in activity from the homogenate assayed immediately after preparation.

Similar results were obtained with cysteine as the reducing agent (Table VI). In this case homogenates containing 100 mg. of tissue per ml. in 0.025 M  $\text{Na}_2\text{HPO}_4$  were prepared from rat heart tissue. Aliquots were exposed to oxygen at 6.9 atmospheres, following which there was, as before, a reactivation period in nitrogen with and without cysteine. The tissues were then assayed by the anaerobic method. In Experiment 2 the activity which had been cut to 32 per cent of the control by high oxygen pressure was completely restored by the treatment with cysteine. In Experiment 1

a lesser amount of cysteine was used during the reactivation period and the reactivation, though only partial, was significant.

*Prevention of Oxygen Inactivation of Succinic Dehydrogenase by Malonate*—Hopkins *et al.* (17) demonstrated that malonate protects succinic dehydrogenase from the inactivation effects of oxidized glutathione. Potter and DuBois (15) also showed this. The latter authors supposed that the free sulfhydryl group of the enzyme is situated between two "affinity points" which serve to bind the substrate for the period of time necessary for the free sulfhydryl group, acting as an oxidation-reduction system, to bring about the oxidation of succinic acid to fumaric acid. The fumarate

TABLE VI

*Reactivation by Cysteine of Succinic Dehydrogenase of Rat Heart Homogenate Preliminarily Inactivated by Oxygen at 7 Atmospheres*

Anaerobic assay method; 30 mg. of tissue used in all assays.

Experiment No.	Preliminary period, 3 hrs., 38°		Reactivation period, 3 hrs., 38°	Subsequent dehydrogenase activity		
	Gas	Pressure	Cysteine added	CO <sub>2</sub> evolved	Succinate oxidized*	Per cent of control
		atmospheres	micromoles per ml.	micromoles per 15 min.	micromoles per gm. per hr.	
1	O <sub>2</sub>	7	0	0.7	47	3
	"	7	50	7.7	520	35
2	N <sub>2</sub>	1	0	22.1	1480	100†
	O <sub>2</sub>	7	0	3.4	170	32
	"	7	100	10.9	545	104
	"	7	100	10.9	545	104
	N <sub>2</sub>	1	0	10.5	525	100†
	"	1	100	11.9	595	113
	"	1	100	12.6	630	120

\* Corrected for the blank in the absence of succinate.

† Control sample.

has a diminished affinity for the enzyme and is displaced by another molecule of succinate. Malonate is similarly but more firmly bound. However, since its structure is such that it is not readily oxidized, it remains to "cover" and protect the active sulfhydryl group between the two affinity points. Hence the addition of oxidized glutathione will have no effect upon the enzyme.

We have demonstrated that malonate similarly protects the enzyme from the inactivating action of high pressures of oxygen. Rat tissue slices were used rather than homogenates because it is easier to remove malonate from them, a necessary step prior to assay. Preliminary experiments showed

that it was possible to immerse slices (0.4 mm.) in phosphate-saline containing 0.025 M malonate for 2 to 3 hours without inactivation. For when the malonate was subsequently removed by washing in the malonate-free medium (three changes, 5 minutes each) and homogenates prepared and assayed by the anaerobic method, the activities were the same as those of slices without malonate treatment. In the experiment (Table VII) the slices were equilibrated for a preliminary period with or without malonate

TABLE VII

*Protection by Malonate (0.025 M) of Succinic Dehydrogenase in Rat Tissue Slices against Inactivating Effect of Oxygen at High Pressures*

Experiment No.	Preliminary period				Assay period	
	Tissue	pO <sub>2</sub> atmospheres	Hrs.	Malonate mM per l.	Succinate oxidized micromoles per gm. per hr.	Per cent inactivation
1	Liver	0.2	2	0	977	
		6.9	2	0	620	37
		6.9	2	25	1040	0
2	"	0.2	2	0	518	
		6.9	2	0	403	22
		6.9	2	25	622	0
3	"	0.2	2	0	328	
		6.9	2	0	183	44
		6.9	2	25	291	11
4	Heart	1 (N <sub>2</sub> )	2.5	0	573	
		1 "	2.5	25	588	0
		6.9	2.5	0	277	52
		6.9	2.5	25	573	0
5	Kidney	0 (N <sub>2</sub> )	2.5	0	585	
		0 "	2.5	25	630	0
		6.9	2.5	0	338	42
		6.9	2.5	25	353	40
6	"	0 (N <sub>2</sub> )	3	0	460	
		0 "	3	25	480	0
		6.9	3	0	140	70
		6.9	3	25	300	35

and with oxygen at 6.9 atmospheres. The malonate was then removed by washing, and the slices were homogenized and assayed. From the data in Table VII it is seen that in all instances but one the presence of the malonate during the preliminary period with high oxygen pressure protected against inactivation. In several instances the protection was complete.

*Protection by Succinate*—It should be expected on the basis of the experiments with malonate that succinate should also prevent the inactivation of

succinic dehydrogenase by high oxygen pressure. Such was found to be the case. The experimental data (Table VIII) show, however, that the succinate concentration must be relatively high and that the protection is only partial. Liver or heart slices were equilibrated during a preliminary period in either saline or 0.1 M succinate solutions at 1 or 7 atmospheres of oxygen. The tissues were then washed in saline, converted into homogenates, and assayed by the anaerobic method. The results were somewhat irregular but there is clear evidence that, compared to the controls, the

TABLE VIII

*Protection by Succinate of Succinic Dehydrogenase in Slices of Rat Tissue against Inactivating Effect of High Oxygen Pressures*

Tissue	Preliminary period		Succinate in preliminary period	Subsequent dehydrogenase activity; succinate oxidized
	O <sub>2</sub>	Hrs.		
	<i>atmospheres</i>		<i>mole per l.</i>	<i>micromoles per gm. per hr.</i>
Liver I	1	2.5	0	312
	1	2.5	0.1	260
	7.2	2.5	0	35
	7.2	2.5	0.1	106
" II	0.2	2.5	0	330
	0.2	2.5	0.2	440
	8.0	2.5	0	245
	8.0	2.5	0.2	433
" III	1.0	3.8	0	84
	1.0	3.8	0.1	214
	7.2	3.8	0	33
	7.2	3.8	0.1	68
Heart I	1.0	3	0	540
	1.0	3	0.1	725
	8.0	3	0	170
	8.0	3	0.1	480
" II	1.0	3	0	350
	1.0	3	0.1	315
	8.0	3	0	115
	8.0	3	0.1	205

presence of 0.1 M succinate diminished significantly the inactivating effects of high oxygen pressure.

*Succinic Dehydrogenase Activity of Brains of Rats Killed by High Oxygen Pressures*—The possibility that the inactivating effect of high oxygen pressure might be greater *in vivo* than *in vitro* was tested. Rats were exposed to 8 atmospheres of oxygen until dead (20 minutes at 8 atmospheres). Following decompression, homogenates were prepared from these brains and assayed anaerobically. For comparison homogenates of brains

from normal rats were similarly assayed. The data are shown in Table IX. It is evident that exposure of 20 minutes to high oxygen pressure sufficient to kill the animal does not inactivate succinic dehydrogenase of the brain. This is in conformity with our experiments *in vitro* which showed that exposures to 7 atmospheres of oxygen for 1 hour or more were required to produced significant inactivation of the enzyme.

*Effect of High Oxygen Pressure on Cytochrome Oxidase*—Heart, liver, or brain tissues from white rats, either as slices or homogenates, were exposed in a preliminary period to high oxygen pressure at 38°. These tissues together with appropriate controls, usually maintained in air, were then assayed for cytochrome oxidase activity by one of the two methods described; viz., (a) the oxygen uptake in presence of *p*-phenylenediamine or (b) oxygen uptake in presence of ascorbic acid. The homogenates were

TABLE IX

*Succinic Dehydrogenase Activity of Brain Homogenates of Rats Killed by Exposure to 7 Atmospheres of Oxygen*

Assay at 38° by anaerobic method; activity calculated from 30 minute assays on 25 and 50 mg. of tissue.

Normal rats		Exposed rats	
Experiment No.	Succinate oxidized	Experiment No.	Succinate oxidized
	<i>micromoles per grs. per hr.</i>		<i>micromoles per grs. per hr.</i>
1	192	1	200
2	228	2	208
3	208	3	192
Mean.....	209 ± 11		200 ± 5

prepared in water and the slices were maintained in customary phosphate-saline. All assays were carried out at 38°. The results of these experiments are given in Table X. In no case were we able to find any effect of high oxygen pressures up to 7 atmospheres for 3 hours on the cytochrome oxidase activities of these tissues.

*Effect of High Oxygen Pressure on Cytochrome c*—The importance of cytochrome *c* in tissue metabolism prompted a study of the effect of high oxygen pressure upon its activity. A solution prepared from horse heart containing 0.38 micromole per ml. was subjected at 38° to 6.9 atmospheres of oxygen for 2 hours. The activity of this preparation compared to that of a control maintained in air for the same length of time was measured as follows: The cytochrome oxidase activity of a rat heart homogenate was measured in the presence of ascorbic acid and cytochrome *c*. Without cytochrome *c* such a preparation is practically inactive. The data of the

TABLE X

*Effect of Oxygen at High Pressure upon Cytochrome Oxidase Activity of Rat Tissues at 38°*

Experiment No.	Tissue	Preliminary period with O <sub>2</sub>		Subsequent cytochrome oxidase activity Oxygen uptake
		atmospheres	hrs.	micromoles per gm. per hr.
I-a	Heart slices	0.2	1	195
		7	1	244
I-b		0.2	3	253
		7	3	213
				168
Assayed at once				
II-a	Liver homogenate	0.2	1	198
		7	1	198
II-b		0.2	1	249
		7	1	268
II-c		0.2	3	114
		7	3	191
III	Heart "	0.2	2*	1920
		0.2	2	1600
		7	2	1520
IV		0.2	1.5*	960
		7	1.5	1200
		7	1.5	1230
V	Brain "	0.2	1.5*	500
		0.2	1.5	610
		7	1.5	550

In Experiments I and II the substrate was *p*-phenylenediamine; in Experiments III to V, ascorbic acid; in Experiments I and II oxygen uptake was corrected for the blank in the absence of substrate; in Experiments III to V oxygen uptake was calculated from the difference obtained with two weights of tissue.

\* Maintained in air at 0°.

TABLE XI

*Effect of Oxygen at High Pressures on Cytochrome c*

Preliminary period, 2 hrs. O <sub>2</sub> pressure	Subsequent cytochrome oxidase activity (0.2 micromole cytochrome c added)		
	Rat heart homogenate	O <sub>2</sub> uptake	Cytochrome oxidase activity; O <sub>2</sub> uptake
atmospheres	mg. tissue	micromoles per 20 min.	micromoles per gm. tissue per hr.
0.2	5	6.0	
0.2	10	8.9	1740
7	5	6.0	
7	10	9.0	1800

assay are given in Table XI. It is evident that cytochrome *c* exposed to high oxygen pressure is just as active in this system as in the control sample kept in air.

#### DISCUSSION

The succinoxidase and cytochrome oxidase systems together account for the major portion of the oxidative metabolism of tissues. It would be expected, then, that any agent having toxic effects upon them should seriously impair the metabolic functions of the intact animal. Many investigators have attempted to show that the poisonous effects of oxygen at high pressure are due primarily to inhibitory effect upon oxidative enzyme systems. This view, pushed to its limit, holds that oxygen poisoning is an acute anoxia due to a rapid inactivation of essential enzyme systems, a state termed "hyperoxic anoxia." We have emphasized in a previous paper (18) that the effects following sudden exposure to oxygen at high pressure (about 8 atmospheres) may be divided into two phases, (a) an acute phase manifested in the intact animal, characterized by convulsions, collapse, and death, and (b) a subacute phase, observed in tissues *in vitro*, characterized by a slow but definite decrease of total metabolism reflected in a decreased oxygen uptake. The time element in this latter phase is so great, relative to that of the acute phase, that it is difficult to believe that generalized enzymatic inactivations can play any rôle in the early death of the animal. This conclusion is well borne out when the total oxygen uptake of intact animals is measured at 8 atmospheres, for in this case the oxygen uptake does not decrease, even though marked symptoms of oxygen poisoning occur, until just before collapse and death.

The experiments reported here on two individual enzyme systems strongly support this view. We have been unable to show any inhibitory action whatever by oxygen at high pressure upon the cytochrome oxidase system. On the other hand the succinoxidase system is inhibited in the course of time, but as a rule very prolonged exposures (1 to 3 hours) are required to produce appreciable decreases. 20 to 25 minutes exposures to 8 atmospheres, which usually kill intact rats with violent symptoms of poisoning, hardly have any effect upon succinoxidase. Furthermore animals killed by oxygen show no significant differences from controls in the activity of succinoxidase prepared from their brains. Metabolic deficiencies due to the inhibition of this enzyme system must therefore be ruled out as a cause of death which follows exposure to high oxygen pressure.

The possibilities that enzymatic inhibitions play a rôle in the case of an animal subjected to a much lower pressure of oxygen but for a longer period of time, *e.g.* 1 atmosphere for 1 to 3 days, must be considered. It is impossible to expose tissues *in vitro* for such prolonged periods. But we have studied the total oxygen uptake of lung slices in dogs exposed for



48 hours to 1 atmosphere of oxygen and have found it to be about normal. However, judgment must be reserved until data on tissues and individual enzymes from such animals are available.

The chemical mechanisms by which oxygen at high pressure produces enzymatic inhibitions are of prime significance in an understanding of the phenomenon. Those thus far proposed in the literature have been reviewed in detail by the present authors (2). The prominence given in the literature to Libbrecht and Massart's *oxygène actif* hypothesis prompted us to test it thoroughly. Experiments of some six different types failed to give any evidence indicating that the cytochrome oxidase system played a rôle in reacting with oxygen at high pressure to inactivate succinic dehydrogenase. We are therefore compelled to conclude that the production of any substance as *oxygène actif* by interaction of oxygen and cytochrome oxidase must be excluded as a cause of oxygen poisoning of succinoxidase.

But in view of Bean and Bohr's (13) recent experiments, it is hazardous to exclude the possibility that a cyanide-sensitive system, which may be cytochrome oxidase, reacts with oxygen at high pressure to cause inactivation of enzymes other than succinoxidase. The mechanism of this hypothetical interaction is unknown and had better not be "fixed" by any appellation, especially such a vague one as "*oxygène actif*."

Bean and Bohr found that the tonus of the pyloric sphincter of the rabbit was rapidly and markedly decreased by oxygen at high pressure. If cyanide was present in the medium, this toxic action of oxygen was eliminated. However, since the tonus itself was unaffected by cyanide alone, they proposed that the metabolism responsible for the maintenance of tonus was dependent upon "dehydrogenases" rather than the succinoxidase system. Oxygen at high pressure reacts with the cytochrome oxidase system, bringing inactivation of these dehydrogenases with resultant loss of tonus. Bean and Bohr assumed that a mechanism similar to that proposed by Libbrecht and Massart was operative here.

It need hardly be emphasized that our experiments are in conflict with those of Libbrecht and Massart, but not with Bean and Bohr's. Obviously further experimentation is needed to clarify the rôle of cyanide-sensitive systems in oxygen poisoning. Until then definitive proposals of mechanisms are premature.

On the other hand our experiments show quite conclusively that the mechanism of inactivation of succinic dehydrogenase is that of oxidation of the active sulfhydryl group to the inactive thiol form. The situation here is essentially that found by Hopkins *et al.* in the inactivation of succinic dehydrogenase by oxidation by oxidized glutathione or cystine. The protection afforded by malonate and succinate against the inactivating oxidation indicates strongly that the hypothesis of Potter and DuBois

that malonate "covers" the —SH group holds with high oxygen as it does with cystine. It is premature to assert that inactivation of enzymes in all cases is due to oxidation of sulfhydryl groups. Other mechanisms may be operative with other enzymes.

The resistance of cytochrome oxidase to oxygen at high pressures is consistent with our general experience with other enzymes; *viz.*, that the susceptibility of enzymes varies greatly, some being easily inactivated, whereas others are completely resistant.

#### SUMMARY

1. Experiments on the effect of oxygen at high pressure on succinic dehydrogenase, cytochrome oxidase, and cytochrome *c* are reported.

2. Oxygen at 7 atmospheres inactivates the succinic dehydrogenase of rat liver, kidney, or brain. The rate of inactivation, however, is relatively slow.

3. The inactivation of cytochrome oxidase by the addition of cyanide does not eliminate the poisonous action of oxygen on succinic dehydrogenase. From this it is concluded that the mechanism of oxygen poisoning is not one in which cytochrome oxidase forms "activated oxygen" which in turn inactivates succinic dehydrogenase.

4. Succinic dehydrogenase inactivated by oxygen at high pressure is reactivated by reduced glutathione or cysteine. The presence of malonate or high concentrations of succinic acid protects the enzyme from the poisonous action of oxygen. The relation of these findings to the mechanism of inactivation by oxygen is discussed.

5. The succinic dehydrogenase activity of brain tissue of rats killed by oxygen at 7 atmospheres is not different from that of normal animals.

6. Cytochrome oxidase and cytochrome *c* are unaffected by oxygen at 7 atmospheres.

7. The significance of these findings in the general problem of oxygen poisoning is discussed.

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## OXYGEN POISONING

### VI. THE EFFECT OF HIGH OXYGEN PRESSURE UPON ENZYMES: PEPSIN, CATALASE, CHOLINESTERASE, AND CARBONIC ANHYDRASE\*

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The possibility that the phenomenon of oxygen poisoning may be explained on the basis of enzyme inactivations by oxygen at high pressure has been discussed since the time of Bert. We have reviewed the literature on this subject (1), and in recent papers have reported on the inhibiting action of high oxygen on certain enzyme systems. But that some enzymes are unaffected by high oxygen was early recognized. Bert (2), for example, reported that salivary diastase, pepsin, invertase, and myrosin were unaffected. In this paper we report on four enzymes: pepsin, catalase, carbonic anhydrase, and cholinesterase. We have been unable to show that exposure to oxygen at 7 to 8 atmospheres has any effect upon their activity.

*Pepsin*—The preparation was a crystalline one obtained from Lehn and Fink. It was dissolved in water but not dialyzed and therefore contained some  $MgSO_4$ . Activity was determined by the method of Riggs and Stadie (3). In essence this method depends upon the photoelectric determination of the rate of decrease of turbidity of a homogenized egg white suspension when acted upon by the enzyme. Activity is expressed in terms of the monomolecular constant of proteolytic digestion. The data of a typical experiment are shown in Table I. Four solutions of pepsin of different concentrations were exposed to 7 atmospheres of oxygen for 2 hours at 38°. Four parallel samples were similarly shaken in air. The samples were then assayed for activity. There was no significant effect upon peptic activity following the exposure to oxygen at high pressure.

*Catalase*—The catalase activity of two different tissue preparations was studied. In addition experiments were done on a purified crystalline catalase which was kindly given to us by Dr. J. B. Sumner of Cornell University. The preparations were exposed in a small pressure chamber at 38° to 7 atmospheres of oxygen for a preliminary period of 4 hours. Follow-

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

ing decompression, they were assayed at room temperature. Parallel samples, serving as controls, were maintained in air (or nitrogen in one case) under the same conditions. The method of assay depended upon the measurement of the rate of decomposition of hydrogen peroxide in the presence of the enzyme. A conventional Warburg respirometer was used. The main vessel contained 1 ml. of 0.25 M sodium phosphate at pH 6.8 and the catalase preparation in a convenient amount. The side sac contained 0.5 ml. of 0.5 M  $\text{H}_2\text{O}_2$ . The total volume was brought to 3.5 ml. The vessels were equipped with manometers and were shaken rapidly in a water bath at 18°. When temperature equilibrium was reached, the contents of the two compartments were admixed, the tap to the manometer closed, and the rate of evolution of oxygen determined manometrically.

TABLE I

*Activity of Pepsin, Catalase, and Cholinesterase Following Preliminary Exposure to 7 Atmospheres of Oxygen*

Enzyme preparation		No. of experiments	Preliminary treatment with oxygen		Subsequent activity  per cent of unexposed control samples
			hrs.	°C.	
Pepsin Catalase	Crystalline	4	2	38	100 ± 0
	"	2	4	38	109 ± 9
	Human red blood cells	2	4	38	102 ± 12
	Rat liver homogenate	3	4	20	100 ± 7
Cholinesterase	" " "	4	4	38	73 ± 3
	Human serum	2	4	38	100 ± 0*
	" "	1	4	38	100†
	Homogenate of rat brain	2	4	38	93 ± 2†

\* Assayed by Method 1.

† Assayed by Method 2.

Rectilinear rates of evolution were obtained and the activity was calculated from that of the first 15 minute period. The data are assembled in Table I. The mean activity compared to the controls is not significantly different from 100 per cent. At 20° there is no evidence that oxygen at 7 atmospheres has any effect upon the catalase activity. At 38° there is some tendency for loss of activity in the liver preparations, but this was not the case with red blood cells or crystalline catalase. It is apparent that catalase is highly or completely resistant to oxygen at 7 atmospheres for 4 hours.

*Cholinesterase*—The striking prominence of symptoms of the central nervous system in oxygen poisoning lends interest to the study of the effect of oxygen at high pressure upon cholinesterase. For this purpose, serum

and homogenates of rat brain were exposed in a preliminary period at 38° to oxygen at 7 atmospheres. Following decompression they were assayed for cholinesterase activity. In all cases parallel samples serving as controls were maintained under identical conditions in air.

*Methods of Assay*—Two methods were used. Method 1 was of the conventional type and depends upon the measurement of the rate of evolution of  $\text{CO}_2$  from a bicarbonate solution due to the hydrolysis of the acetylcholine by the enzyme. The conventional Warburg vessel equipped with a manometer was used. The main vessel contained the enzyme system in 0.03 M  $\text{NaHCO}_3$ . The side sac contained 0.5 ml. of 2.5 per cent acetylcholine. The total volume was adjusted to 3.5 ml. with NaCl solution sufficient to make a final concentration of 0.1 M. The gas phase was 5 per cent  $\text{CO}_2$  and 95 per cent  $\text{N}_2$ . The vessel was shaken in a water bath at 38° and when temperature equilibrium was reached the contents of the two compartments were admixed and the tap to the manometer closed. The rate of evolution of  $\text{CO}_2$  was determined for the period 5 to 35 minutes after mixing.

Method 2 allowed of the determination of esterase activity *during* the period of high oxygen pressure. It depends upon the fact that acid formation by hydrolysis of the acetylcholine changes the pH of the phosphate medium. The change can be measured by the glass electrode. From this and the composition of the medium it is possible to calculate the rate of hydrolysis of the acetylcholine. 1 ml. of serum was added in a small Erlenmeyer flask to 6 ml. of a buffer solution of the following composition:  $\text{Na}_2\text{HPO}_4$  0.080 M,  $\text{NaH}_2\text{PO}_4$  0.020 M, NaCl 0.010 M. To this was quickly added 1 ml. of a 2.5 per cent acetylcholine solution in 0.150 M NaCl. A sample was immediately removed and the pH rapidly determined. The flask was then quickly brought to 7 atmospheres of oxygen in a small pressure chamber containing water at 38°. Shaking was started and at the end of 1 hour the pH of the reaction mixture was again determined. The acid formation was calculated and expressed as microequivalents per ml. The results of the experiments are given in Table I. It is obvious that whether the activity is measured during exposure to high oxygen or following a period of 4 hours exposure there is no effect of oxygen at 7 atmospheres upon the enzymatic cholinesterase activity of serum or brain homogenate.

It has been shown by Nachmansohn and Lederer (4) and Barron and Singer (5) that the activity of cholinesterase depends upon free —SH groups. We have previously shown (6), in the case of succinic dehydrogenase, that oxygen at high pressure inactivates the enzyme by oxidizing the sulfhydryl group. Its failure to do so in the case of cholinesterase requires comment. It is of course possible that the use of higher pressures

of oxygen for a longer period of time than were employed by us might have brought about inactivation by this mechanism. However, Barron has emphasized the fact that —SH groups of enzymes vary considerably in their susceptibility to oxidizing or other reagents. This apparently applies to oxygen as well, and cholinesterase is an example of a sulfhydryl enzyme resistant to oxygen at high pressure. In other words the fact that an enzyme is of the sulfhydryl type need not signify that its activity will be inhibited by oxygen.

That the —SH group in cholinesterase is resistant to oxidizing agents other than oxygen is brought out by the following experiment. The amount of free —SH groups present in human serum was determined by the method of Helleman, Chinard, and Ramsdell (7). Sufficient *o*-iodosobenzoate was then added to the serum to oxidize to 100 or 200 per cent of the total free —SH groups. The results compared to controls are given in Table II. It is apparent that *o*-iodosobenzoate even in amounts twice that necessary to oxidize all —SH groups in the serum has little or no

TABLE II  
*Effect of o-Iodosobenzoate upon Cholinesterase Activity of Human Serum*

<i>o</i> -Iodosobenzoate added	Per cent of sulfhydryl groups oxidized	Esterase activity as acid formation
<i>micromoles per ml.</i>		<i>microequivalents per ml. per hr.</i>
0	0	72
3.9	100	70
7.8	200	67

effect upon the cholinesterase activity. This observation is consistent with the enzyme's resistance to high oxygen pressure and the properties of other —SH enzymes, some of which are inactivated with difficulty by sulfhydryl reagents (*cf.* Barron and Singer (8)).

**Carbonic Anhydrase**—Disturbed transport of CO<sub>2</sub> by the blood as a factor in oxygen poisoning has been reviewed by Stadie, Riggs, and Haugaard (1) and by Bean (9). Carbonic anhydrase plays an important rôle in this transport and for these reasons we determined the anhydrase activity of red blood cells before and after exposure to 8 atmospheres of oxygen.

**Method of Assay**—Red blood cells were washed twice with saline. The packed cells were diluted 1:10 with water and after 10 minutes the solution was centrifuged heavily. The supernatant solution was used for the experiments.

The assay was carried out at 5° in a Warburg vessel equipped with a manometer. The vessel was also equipped with a hollow side stop-cock which contained 1.0 ml. of 0.5 M phosphate at pH 6.8. This could be

admitted into the main vessel at the beginning of the assay. The main vessel contained 1.0 ml. of 0.025 M  $\text{NaHCO}_3$  in 20 per cent glucose. The glucose was used to give the contents of the main vessel a high density. In consequence, the contents of the side stop-cock when admitted floated on top until the shaking was begun. The shaking was rapid and control experiments showed that it was sufficient to eliminate slow physical diffusion of  $\text{CO}_2$  out of the aqueous phase as a cause of error. Upon admixture of the reagents by shaking, readings were begun. Since all assays were made under the same conditions, the results are expressed in terms of changes of level of manometer fluid in mm. per second.  $\text{H}_2\text{CO}_3$  is formed instantaneously upon the mixing of the bicarbonate and acid

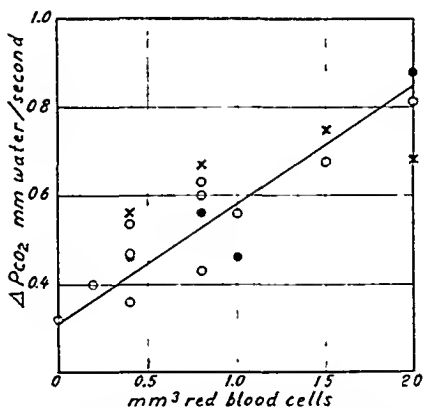


FIG. 1. Effect of preliminary exposure to high pressure of oxygen at 38° on the carbonic anhydrase activity of red blood cells. O unexposed sample. ● sample exposed to 8 atmospheres of oxygen for 1 hour; X sample exposed to 8 atmospheres of oxygen for 2 hours.

phosphate. The  $\text{H}_2\text{CO}_3$  then breaks down to  $\text{CO}_2$ , the rate being dependent upon the amount of carbonic anhydrase catalyzing the reaction. The results of the experiment are shown in Fig. 1. The following points are noted. (1) The velocity of dehydration of  $\text{H}_2\text{CO}_3$  as measured by the rate of evolution of  $\text{CO}_2$  is a rectilinear function of the amount of carbonic anhydrase added in the red blood cells. (2) 1 or 2 hours preliminary exposure to 8 atmospheres of oxygen have no effect upon the carbonic anhydrase activity.

#### DISCUSSION

The finding that some enzymes are completely or highly resistant to the action of oxygen at high pressure increases the probability that the inhib-



itory action upon the susceptible ones is due to some specific type of oxidizing reaction dependent upon the structure of the active group of the enzyme. The various types of inactivating reactions which have been already more or less defined have been discussed by us (1). The fact that one type of active group reacts with oxygen in one enzyme so that inhibition of activity occurs is no indication, however, that the same group in another enzyme will react similarly. This is illustrated by our experience with cholinesterase. Although it is a sulfhydryl enzyme, it is resistant to oxygen at high pressure, unlike other sulfhydryl enzymes, for example succinic dehydrogenase.

It is to be further noted that three of the oxygen-resistant enzymes reported upon in this paper as well as those discussed by Bert, *viz.* pepsin, salivary diastase, invertase, myrosin, cholinesterase, and carbonic anhydrase, are enzymes which catalyze the addition or subtraction of water. We know of no reports showing that such hydrolytic enzymes are affected by oxygen. In the case of urease and arginase (1) the inactivation observed with crude preparations in the presence of oxygen is eliminated by further purification of the enzyme. On the basis of this, one might draw the general conclusion that the active group of enzymes of this type is of a character resistant to the oxidizing action of oxygen at high pressures. In contrast, many enzymes concerned with oxidation-reductions are susceptible.

#### SUMMARY

1. The enzymatic activities of preparations containing pepsin, catalase, cholinesterase, and carbonic anhydrase were determined before and after exposure to oxygen at high pressure. No significant changes were observed.

2. Cholinesterase is an example of a sulfhydryl enzyme unaffected by oxygen.

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## OXYGEN POISONING

### VII. THE EFFECT OF HIGH OXYGEN PRESSURE UPON ENZYMES: URICASE, XANTHINE OXIDASE, AND *d*-AMINO ACID OXIDASE\*

By WILLIAM C. STADIE AND NIELS HAUGAARD

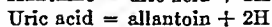
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(Received for publication, August 21, 1945)

As part of a general study of the problem of oxygen poisoning experiments on the effect of oxygen at high pressure upon individual enzyme systems have been reported (1). Further studies are reported here on three enzymes. Uricase and xanthine oxidase were selected because they were concerned with purine metabolism. *d*-Amino acid oxidase, though of problematical significance in mammalian metabolism, is of interest because it is a well studied enzyme representing the flavin enzyme group. The method of study was similar to that hitherto used. Tissue slices or homogenates were exposed to elevated pressures of oxygen in a preliminary period. They were then assayed for enzyme activity in a subsequent period. Tissue from white rats was used throughout.

#### *Effect of Oxygen upon Uricase and Xanthine Oxidase*

Both of these enzymes are abundant in homogenized preparations of rat liver; hence it was convenient to study them together. Upon equilibration of the tissue with xanthine, the oxidation proceeds in two steps to allantoin, viz.



Since it was shown in all assays for xanthine oxidase activity that uricase activity was not a limiting factor, it is possible to assume that 1 mole of oxygen corresponds to the oxidation of 1 mole of xanthine. On the other hand, when uric acid is the substrate, 1 mole of oxygen corresponds to the oxidation of 2 moles of uric acid. All activities were calculated accordingly.

*Method of Assay*—The method is essentially that of Axelrod and Elvehjem (2). Instead of a differential manometer, two Warburg respirometers were used, one with and the other without xanthine. The main compart-

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

ment of the respirometer contained a 1:10 liver homogenate with 200 to 300 mg. of tissue. The side sac contained 0.15 ml. of 0.050 M neutral xanthine solution (except in the blanks). The center well was equipped with an alkali filter roll inset to absorb the  $\text{CO}_2$  formed. 20 minutes after temperature equilibrium had been reached ( $38^\circ$ ), the contents of the two compartments were admixed and the oxygen uptake measured against time. After the first 5 minutes a rectilinear rate was usually observed for the subsequent 40 minutes. The rate with xanthine was corrected by subtracting the rate without xanthine. From this was calculated the rate of xanthine oxidation in micromoles per gm. of tissue per hour. The rate of uric acid oxidation was also determined. In all cases this was so great in comparison with the xanthine oxidation that it was possible to

TABLE I

*Uricase and Xanthine Oxidase Activity of Rat Liver Homogenate Following Preliminary Exposure at  $38^\circ$  to Oxygen at 7 Atmospheres*

Preliminary period		Subsequent activity			
atmospheres $\text{O}_2$	hrs.	Urate oxidized		Xanthine oxidized	
		micromoles per gm per hr.	per cent of control in $\text{N}_2$	micromoles per gm. per hr.	per cent of control in $\text{N}_2$
0.2	2	80	107	25.1	91
		79	90	28.5	98
1	2	80	107	12.2	45
		71	81	8.3	29
		98	83	25.0	70
7	2	59	87	2.8	9
		106	90	2.2	6
7	4	68	113	0.0	0
Mean.		95			

assume that each mole of oxygen uptake corresponded to the oxidation of 1 mole of xanthine, according to the above equation.

The uricase activity was determined in the same way. In this case the side sac contained 0.3 ml. of 0.04 M sodium urate to give a final concentration of 0.006 M.

*Experimental Results*—Preliminary experiments showed that xanthine oxidase was stable in liver homogenate for 48 hours at  $0^\circ$ . The activity was independent of the concentration of xanthine when this was varied from 0.0013 to 0.0075 M. In Table I are assembled the data showing the effects of oxygen. In all cases parallel samples maintained in 1 atmosphere of  $\text{N}_2$  were used for comparison. In no cases were we able to show that oxygen at pressures up to 7 atmospheres for 4 hours had any effect upon

the uricase activity of the homogenate. On the other hand, xanthine oxidase is very sensitive to oxygen. Oxygen at 1 atmosphere for 2 hours was sufficient to decrease the activity to 29 to 70 per cent of the control. At 7 atmospheres the inhibition was practically complete in 2 hours and entirely so in 4 hours. The sensitivity of xanthine oxidase to oxygen is brought out by the data shown in Fig. 1. Here are plotted the ratios of activities of a liver homogenate in oxygen and air. While the activity in air is well maintained, that in oxygen falls off rapidly to half the control value in about 40 minutes. Complete inactivation was observed in about 2 hours.

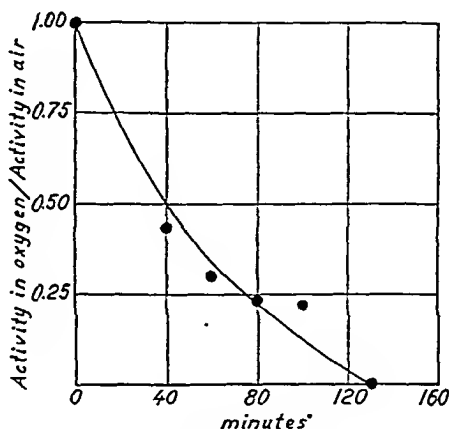


FIG. 1. The ratio of the xanthine oxidase activity of rat liver homogenate in 1 atmosphere of oxygen and in air as a function of time.

*Inactivation of Xanthine Oxidase in Tissue Slices*—Slices of rat liver in buffered medium were exposed to 7 atmospheres of oxygen for 3 hours at 38°. They were then homogenized and assayed for xanthine activity. The data are collected in Table II. Compared to the control samples, the xanthine oxidase activity fell to a very low level following the exposure. In two instances (choline acetylase and *D*-amino acid oxidase) we have found that the enzyme activity of slices of tissue was unimpaired by 4 hours exposure to 7 atmospheres of oxygen, whereas inactivation is rapid when the homogenized tissue is exposed. In the case of xanthine oxidase the enzyme was found to be approximately equally susceptible to oxygen whether the tissue was in the form of a homogenate or in slices.

*Possible Formation of Inhibitor of Xanthine Oxidase by Oxygen*—We have previously discussed (3) the possibility that exposure of tissues to oxygen at

high pressure may result in the formation of inhibitors which specifically inhibit the action of certain enzymes. This possibility was investigated in the case of xanthine oxidase as follows: Rat liver homogenate was exposed to oxygen at 7 atmospheres for  $2\frac{1}{2}$  hours. Its xanthine oxidase activity was reduced to approximately one-third of the unexposed tissue. The original active and exposed inactivated samples were then mixed in

TABLE II  
*Inactivation of Xanthine Oxidase in Liver Slices by Exposure to Oxygen at 7 Atmospheres*

Preliminary period, 3 hrs.	Subsequent activity; xanthine oxidation  micromoles per gm. per hr.
Homogenate assayed at once.....	26.0
Slices kept in saline at room temperature.....	21.4
" " " " " 38°.....	18.0
" in saline exposed to 7 atmospheres of O <sub>2</sub> at 38°.....	2.8

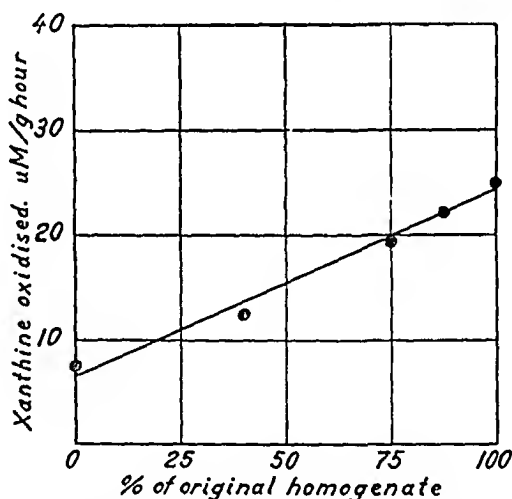


FIG. 2. Xanthine oxidase activity of mixtures of active and oxygen-inactivated (7 atmospheres) rat liver homogenate.

various proportions and assayed. The data are shown in Fig. 2. The activities of the mixtures were found strictly additive, as is shown by the rectilinear relation between activity and the proportions of the two samples. In other words, there was no evidence that the inactivated homogenate contained any substance which inhibited the activity of the unexposed homogenate.

*Effect of Oxygen upon d-Amino Acid Oxidase of Rat Kidney*

*d*-Amino acid oxidase was selected as a representative flavin enzyme. It was studied in homogenates or slices, rat kidney being selected as the source of the enzyme. These preparations were subjected to the action of oxygen in a preliminary period, following which they were assayed for activity. Suitable controls were run in parallel.

*Method of Assay*—Homogenates of fresh rat tissue were prepared by the Potter-Elvehjem method in distilled water at 0°. 1 ml. contained 100 mg. of tissue. Tissue slices were prepared by the Stadie-Riggs (4) method. The *d*-amino acid oxidase activity was determined by measuring the rate of oxygen uptake in the presence of *dl*-alanine or valine. The conventional Warburg vessel was used, the main compartment of which contained the tissue and 1 ml. of 0.125 M sodium pyrophosphate at pH 8.4. The side sac contained 0.25 ml. of 1.0 M *dl*-alanine or valine and 0.1 ml. of a solution of partially purified flavin adenine dinucleotide. The final volume was 2.5 ml. Glass-distilled water was used throughout. When temperature equilibrium was reached (38°), the contents of the compartments were admixed, the tap to the manometer closed, and readings begun. The rate of oxygen uptake was usually uniform for a period of 20 to 30 minutes and the activity of the preparation was calculated from this in terms of micromoles of oxygen per gm. of tissue per hour. With homogenates, the oxygen uptake in the absence of substrate was so small that it could be neglected. With tissue slices, parallel samples of tissue equilibrated without substrate were also run. The oxygen uptake of these samples was used as a blank to correct that found in the presence of substrate. The difference is assumed to measure the *d*-amino acid activity. Unless an inhibitor of "endogenous" oxygen uptake is used, the blank with slices is large. To diminish this blank arsenite at 0.005 M was used. Experience has shown that this is a reliable method for determining *d*-amino acid oxidase activity of slices.

*Effect of Oxygen on Homogenates*—Experience soon showed that *d*-amino acid oxidase in homogenates is so sensitive to oxygen that the effect can be demonstrated by subjecting the homogenate in a preliminary period to air at 38°. The data are assembled in Table III.

The following points are to be noted: (1) When the homogenate is maintained *in vacuo* at 38° for 2 hours, the loss of *d*-amino acid oxidase activity determined in the subsequent assay was 20 per cent and about equal to the loss of the sample maintained in air at 0°. (2) Samples equilibrated at 38° with N<sub>2</sub> at 1 atmosphere lost activity, but those samples equilibrated in air decreased to a significantly greater extent. The loss of activity in nitrogen might possibly be explained by assuming that small amounts of oxygen were still present in the system.

TABLE III

*Effect of Oxygen upon d-Amino Acid Oxidase Activity of Rat Kidney Homogenate*

Experiment No.	Preliminary period, 2 hrs.		Subsequent activity	
	Gas phase	Temperature	O <sub>2</sub> uptake (20 min.; 100 mg. tissue)	
		°C.	micromoles per gm. per hr.	per cent of control
I	Assayed immediately		158	Control
	Vacuum	38	126	80
	Air	0	138	87
	"	38	24	15
II	Assayed immediately		114	Control
	Air	0	114	100
	N <sub>2</sub>	38	48	42
	Air	38	30	26
III	Assayed immediately		166	Control
	N <sub>2</sub>	38	59	36
	Air	38	32	19
IV	Assayed immediately		235	Control
	N <sub>2</sub>	38	118	50
	Air	38	32	14
V	Assayed immediately		130	Control
	N <sub>2</sub>	38	35	27
	Air	38	9	7

Experiments I, III, IV, and V, substrate *dl*-alanine; Experiment II, *dl*-valine. Oxygen uptake with no substrate essentially 0.

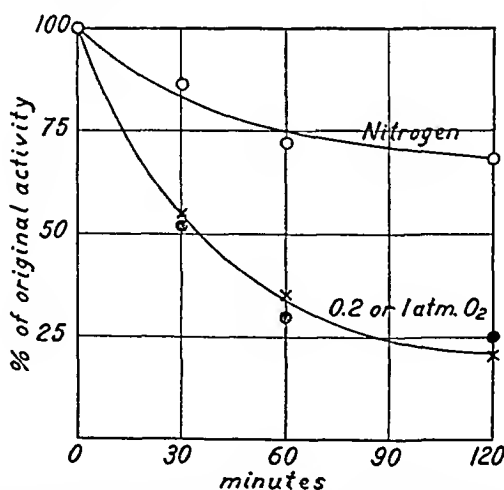


FIG. 3. Effect of preliminary exposure at 38° to nitrogen, air, or 1 atmosphere of oxygen upon the *d*-amino acid oxidase activity of rat kidney homogenate.

The loss of *d*-amino acid activity in the presence of  $N_2$  occasioned some doubt as to the reality of the greater loss in the presence of oxygen. The reality of the effect of oxygen, however, is demonstrated by the data shown in Fig. 3. Rat kidney homogenate was exposed in a preliminary period at  $38^\circ$  to oxygen for varying lengths of time and then assayed. The loss of activity in nitrogen is apparent as before, but there can be no doubt that the loss in air is significantly greater and more rapid. In this case, in-

TABLE IV

*Effect of Oxygen upon d-Amino Acid Oxidase Activity of Rat Kidney Homogenate in Presence of Crystalline Catalase*

Preliminary period, 2 hrs. in air at $35^\circ$ Catalase added		Subsequent activity; <i>dl</i> -alanine as substrate	
$\gamma$ per ml.		O <sub>2</sub> uptake	
		micromoles per gr. per hr.	per cent of control
0		12	7
0		15	9
320		12	7
320		12	7
Assayed immediately		161	Control

TABLE V

*Effect of Oxygen upon d-Amino Acid Oxidase Activity of Rat Kidney Slices*

Experiment No.	Preliminary period, 2 hrs. at $38^\circ$		Subsequent activity	
			Excess O <sub>2</sub> uptake with valine	
		atmospheres	micromoles per gr. per hr.	per cent of control
I	N <sub>2</sub>	1	103	Control
	O <sub>2</sub>	0.2	97	94
	"	7	79	82
II	N <sub>2</sub>	1	107	Control
	O <sub>2</sub>	7	98	91

creasing the oxygen tension from 0.2 atmosphere (air) to 1 atmosphere did not enhance the degree or rate of inactivation.

It is known that hydrogen peroxide is formed when an amino acid is oxidized by oxygen in the presence of *d*-amino acid oxidase. It is conceivable that sufficient excess of hydrogen peroxide may form in the homogenate and inactivate the enzyme. No support was found for this hypothesis in the experiment shown in Table IV, in which a very large excess of crystalline catalase was added to the kidney homogenate during the preliminary period of 2 hours exposure at  $38^\circ$  to air. It was assumed that under these circumstances no excess of  $H_2O_2$  could form. Subse-



quent assay showed that loss of activity was practically complete irrespective of whether added catalase was present or not.

*Effect of Oxygen on Tissue Slices*—We have noted in the case of choline acetylase that the enzyme is extremely susceptible to oxygen in homogenized tissue, but completely resistant when the tissue is exposed in the form of slices. The same observation was made in the case of *d*-amino acid oxidase. Rat kidney slices were exposed at 38° in a preliminary period to oxygen up to 7 atmospheres for 2 hours. They were then assayed for *d*-amino acid oxidase activity with *dl*-valine as substrate. In all cases control slices were assayed in the absence of substrate to determine the blank "endogenous" oxygen uptake. This ranged from 15 to 20 per cent of the oxygen uptake with substrate. All assays were carried out in the presence of 0.005 M arsenite to diminish the value of the blank. The data of the experiment are shown in Table V. It is seen that the ability of the slices to oxidize *dl*-valine was not significantly impaired by the preliminary exposure to oxygen at 7 atmospheres.

#### SUMMARY

1. The effect of a preliminary exposure of rat tissue to oxygen at high pressure upon the subsequent uricase, xanthine oxidase, and *d*-amino acid oxidase activities was studied.
2. Uricase is unaffected by 4 hours exposure to 7 atmospheres of oxygen.
3. Xanthine oxidase, in either homogenized or sliced tissue, is rapidly inactivated by oxygen.
4. *d*-Amino acid oxidase in homogenized tissue is very susceptible to oxygen. In tissue slices, however, it appears to be unaffected by exposures of 2 hours to 7 atmospheres of oxygen at 38°.

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## OXYGEN POISONING

### VIII. THE EFFECT OF HIGH OXYGEN PRESSURE ON ENZYMES: THE SYSTEM SYNTHESIZING ACETYLCHOLINE\*

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Man and experimental animals when subjected to elevated pressure of oxygen quickly show symptoms of nervous origin. For example, rats when exposed to 8 atmospheres of oxygen develop within a few minutes uneasiness, twitching, tonic and clonic spasms, opisthotonus, body rigidity, and generalized convulsions. Within 15 to 25 minutes collapse and death follow. We have already pointed out (6) that these symptoms are manifestations of the *acute phase* of oxygen poisoning. In a systematic study we have been unable to show that during this phase there is any decrease in the activity of the enzymatic systems thus far studied. Nor is there any significant decrease in the total oxygen uptake, until just before death of the animal. In other words, no outstanding disturbances of metabolic activity were demonstrable. On the other hand, what we call the *subacute phase* is characterized by a slow decrease of activity of several important metabolic enzymes demonstrable by studies of tissues and enzymes *in vitro*. But the time element in these two phases is decidedly different. The acute phase develops within a few minutes; the subacute phase requires  $\frac{1}{2}$  to 2 hours for development.

In consequence of these findings, the exact part played by enzyme inhibition in the phenomenon of oxygen poisoning is problematical. It appears safe to conclude that there is at present no proof that such inhibitions are the fundamental cause of the remarkable series of ill events which follows *immediately* upon exposure to high oxygen pressures. In other words, the exact nature of the rapidly developing mechanisms terminating in death remains a matter of speculation.

In a discussion of mammalian nervous reactions, acetylcholine occupies a major position. We have already shown (7) that cholinesterase is completely resistant to oxygen at high pressures. There remained to be tested the obvious possibility that the enzyme systems concerned in the synthesis of acetylcholine might be peculiarly susceptible. In that event,

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

sudden cessation of acetylcholine synthesis upon exposure to elevated pressures of oxygen might conceivably explain the severe ensuing train of nervous symptoms.

In the experiments here reported, the general method hitherto used was followed: The enzymatic activity of tissues *in vitro* was determined either during or after exposure to pressures of oxygen at 7 to 8 atmospheres. In all cases the white rat was used as a source of material.

The experiments reported are of two types.

It has been known since the work of Quastel, Tennenbaum, and Wheatley (3) that slices, minces, or homogenates of brain when equilibrated aerobically in a medium containing glucose will synthesize acetylcholine. Accordingly synthesis under these circumstances was measured during exposure to high pressures of oxygen. Samples at 1 atmosphere of oxygen served as controls.

Nachmansohn and Machado (2) have demonstrated that there is an enzyme in brain, choline acetylase, which is extremely active in synthesizing acetylcholine. With extracts or homogenates of brain as the source of the enzyme and adenosine triphosphate to furnish energy-rich phosphate, it is possible to demonstrate under *anaerobic* conditions a high rate of synthesis, approximately 10 to 15 times that observed aerobically with slices of brain. Apparently the potential capacity of the enzyme greatly exceeds that used for normal function, a finding in general accord with experiences with other enzyme systems. Presumably Nachmansohn and Machado's anaerobic method gives a better indication of the total choline acetylase content of the tissue than does the aerobic method employing the slice. Accordingly, slices were exposed to high oxygen pressure in a preliminary period and, after conversion to homogenates or extracts, assayed for choline acetylase activity. Control experiments showed that this method gave reliable measurements of the enzyme activity of slices of brain. In addition, extracts of brain were exposed in a preliminary period and their subsequent activity measured.

In all experiments measuring synthesis eserine or physostigmine must be present in suitable concentration. Otherwise, acetylcholine will be rapidly hydrolyzed by the cholinesterase of the tissues and no accumulation will occur.

### Methods

*Experiments with Slices*—0.35 or 0.5 mm. slices of brain of large white rats were prepared by the tissue microtome of Stadie and Riggs (5). The suspending medium had the following composition: 0.070 M NaCl, 0.005 M KCl, 0.030 M  $\text{Na}_2\text{HPO}_4$ , 0.010 M  $\text{NaH}_2\text{PO}_4$ , 0.002 M  $\text{MgCl}_2$ , choline 0.005 M, 0.4 per cent glucose, eserine 1:10,000. The slices from one brain

(600 to 900 mg.) were divided equally between two small Erlenmeyer flasks containing 3 ml. of the medium kept at 0° until the beginning of the experiment. The gas phase was oxygen at 1 atmosphere or 8 atmospheres, a small pressure chamber being used in the latter case. The flasks were brought to 38° and gently shaken in a water bath. One sample was prepared at once for analysis to give the initial value; the second was similarly treated at the end of the experimental period. The difference between these two values gives the amount of acetylcholine synthesized.

*Preparation for Analysis*—Minor modifications occurred during the course of the work, but in principle the method was as follows: It has been abundantly shown (*e.g.* (1)) that acetylcholine exists in two forms, (a) free and hence demonstrable in the medium, and (b) bound (presumably with protein) and hence not demonstrable unless released by appropriate methods. Accordingly, the medium from the slices was filtered off and analyzed separately. The slices containing the bound acetylcholine were transferred to a solution of 0.005 M HCl, 0.005 M  $\text{Na}_2\text{HPO}_4$ , and eserine 1:50,000. The slices were then homogenized in this medium with the Potter-Elvehjem homogenizer and allowed to stand at room temperature for 1 hour. The sample was then placed in boiling water for 2 minutes, centrifuged, and the sedimented tissue washed twice with the acid medium. Supernatant and washings were carefully neutralized, made up to 10 ml., and kept at 0° until analyzed. In later experiments, the determination of free and bound acetylcholine was discontinued because we were unable to demonstrate any need for the separate determinations. In this case the original medium was brought to 0.005 M with respect to HCl and the method pursued essentially as described with tissue above.

Many control experiments in which known amounts of acetylcholine were added to tissue were done to test the various steps in the above method of preparation. The results convinced us that the method was sound.

*Experiments with Homogenate of Brain*—The methods were essentially the same as with slices. In the preparation of the tissue for analysis the medium was adjusted to pH 5.5 by the addition of an acetate buffer. To release bound acetylcholine the heating at 100° was continued for a period of 40 minutes. As before, all steps were controlled by recovery experiments with known amounts of added acetylcholine.

Whereas the acidification of the tissue is necessary to release bound acetylcholine, the necessity of heating, particularly at pH 2, is in question. On this point we followed the method of Abdon and Hammarskjöld (1), but some control experiments indicated that at the more acid reaction the heating may safely be omitted.

*Anaerobic Assay of Choline Acetylase*—The method was essentially that of Nachmansohn and Machado (2). This requires the preparation of a

cell-free extract of tissue which is subsequently assayed for enzymatic activity. Weighed samples of rat brain were homogenized with the aid of a small amount of fine quartz powder in 5 parts of the following medium: 0.065 M sodium phosphate (pH 7.0), 0.05 M KCl. The tissue suspension was centrifuged at 2000 R.P.M. for 3 minutes and then filtered through gauze. The reaction was carried out in Thunberg tubes. 1 ml. of the extract was placed in each tube. The side compartment contained 0.25 ml. of a solution sufficient to give concentrations in the final reaction mixture approximating 0.02 M sodium acetate, 0.03 M sodium fluoride, 0.06 per cent physostigmine sulfate, 0.002 M choline chloride, and 0.006 M adenosine triphosphate. The tube was then thoroughly evacuated and placed in a water bath at 37°. After 3 minutes, the reaction was started by admixture of the contents of the two compartments. After 15 minutes, the reaction was terminated by the addition of 0.5 ml. of 0.3 M HCl in 0.07 M phosphate. After 30 minutes at room temperature, the solution was neutralized, made up to volume, and analyzed for acetylcholine.

*Determination of Acetylcholine*—The contractile response of the frog rectus abdominis was used. The suspending medium of frog-Ringer-bicarbonate contained eserine 1:50,000. The muscle lever was a high inertia type designed by Riggs (4). The magnification was approximately 20. A kymograph was dispensed with, the pointer readings being read on a scale. Samples of fixed volume were added to the medium and the change of reading of the pointer recorded after 5 minutes. The muscle was then washed with fresh medium and allowed to relax to the original position (5 to 15 minutes) before a new determination was made. Each muscle was standardized with known amounts of acetylcholine and the unknown samples were interspersed with known samples. As a rule, closely agreeing results were obtained with different muscles. Under the conditions selected amounts of acetylcholine from 0.2 to 0.6  $\gamma$  could be determined with an error not exceeding 5 per cent.

### Results

*Aerobic Synthesis of Acetylcholine by Rat Brain Slices*—A comparative study of the rate of synthesis under 1 and 8 atmospheres was made. The methods used were those already outlined. The data are given in Table I. With the exception of two aberrant results, the synthesis of acetylcholine at 1 atmosphere of oxygen by slices of rat brain tissue was reasonably uniform and approximately of the order found by Quastel *et al.* (3). When the slices are equilibrated in 8 atmospheres of oxygen, no significant difference in rate of synthesis was found. In four experiments free and bound acetylcholine was determined separately, but the results are combined in Table I because the division failed to show anything of significance.

Apparently the system of enzymes synthesizing acetylcholine in slices of brain is resistant to oxygen at 8 atmospheres for a period of 1 hour.

*Aerobic Synthesis by Brain Homogenates*—With homogenates, the aerobic synthesis of acetylcholine by parallel samples at 1 and 8 atmospheres of

TABLE I  
*Aerobic Synthesis of Acetylcholine by Slices of Rat Brain during 1 Hour's Exposure at 38° to 1 or 8 Atmospheres of Oxygen*

Medium, saline-phosphate (pH 7.4) + 0.2 per cent glucose. The results are expressed in micrograms per 100 mg. of brain.

O <sub>2</sub> , 1 atmosphere				O <sub>2</sub> , 8 atmospheres			
Rat No.	Initial	Final	Synthesis	Rat No.	Initial	Final	Synthesis
1	4.3	10.6	6.3	10	4.7	14.4	9.4
2	4.9	11.4	6.5	11	8.7	25.5	16.8
3	8.8	21.3	12.5	12	7.2	22.5	16.0
4	3.8	18.8	15.0	13	5.3	10.7	5.4
5	4.0	6.7	2.7	14	4.2	11.2	7.0
6	4.2	12.2	8.0	15	5.0	10.4	5.4
7	4.3	3.8	-0.5*	16	2.8	12.8	10.0
8	12.1	10.2	-1.9*	17	3.1	11.5	8.4
9	3.3	13.0	9.7	18	5.1	7.7	2.6
Mean $\pm$ standard error of mean.....8.7 $\pm$ 1.6				9.0 $\pm$ 1.7			

\* Excluded from the mean.

TABLE II  
*Aerobic Synthesis of Acetylcholine by Homogenates of Rat Brain during Exposure at 38° to 1 or 8 Atmospheres of Oxygen*

Medium, saline-phosphate (pH 7.4) + 0.2 per cent glucose.

Homogenate No.	Exposure  hrs.	Acetylcholine synthesis, per 400 mg. tissue	
		O <sub>2</sub> , 1 atmosphere	O <sub>2</sub> , 8 atmospheres
1	1	4.6	4.1
2	1	4.2	4.3
3	2	4.0	4.0
4	3	4.6	4.4

oxygen could be compared. Exposures were extended up to 3 hours. The results of the experiments shown in Table II confirm the results with brain slices. There was no decrease in synthesis at the higher pressure of oxygen.

*Choline Acetylase*—The aerobic synthesis of acetylcholine in intact tissue is presumably due to a complex of enzymes. In contrast, choline acetylase is presumably a single enzyme which will catalyze the synthesis of acetylcholine from choline and acetic acid anaerobically provided adenosine triphosphate is present to serve as a donor of energy-rich phosphate. By the method of Nachmansohn and Machado already outlined, an estimation of the acetylase activity of brain slices can be made. In control experiments different weighed slices from the same brain were converted to extracts and then assayed according to the method. Substantial concordance was found and it was thus possible to treat brain slices by

TABLE III

*Effect of Preliminary Exposure to 8 Atmospheres of Oxygen upon Subsequent Choline Acetylase Activity of Slices of Rat Brain*

Rat No.	Preliminary period		Subsequent anaerobic choline acetylase activity; acetylcholine synthesized per gm. tissue per hr.
		min.	$\gamma$
1	Vacuum, 38°	15	69
	O <sub>2</sub> , 1 atmosphere, 38°		86
	" 8 atmospheres, 38°		86
2	Vacuum, 38°	30	58
	O <sub>2</sub> , 1 atmosphere, 38°		30
	" 8 atmospheres, 38°		37
3	Vacuum, 0°	30	72
	O <sub>2</sub> , 1 atmosphere, 38°		45
	" 8 atmospheres, 38°		57
4	Vacuum, 0°	30	92
	" 38°		52
	O <sub>2</sub> , 1 atmosphere, 38°		64
	" 8 atmospheres, 38°		82

different methods and subsequently assay them for enzyme activity. Accordingly slices were maintained *in vacuo* in 1 or 8 atmospheres of oxygen for varying periods of time and then assayed anaerobically. From the experimental results assembled in Table III it is again apparent that this more direct method of measuring specific enzyme activity fails to show any effect of oxygen at high pressure upon activity.

The situation was found to be different when the enzyme in extracts prepared as described under "Methods" was exposed to high oxygen pressure. Under these circumstances, the enzyme is quite susceptible to oxygen, activity being rapidly lost, as the data collected in Table IV show. This observation is not unique and has been encountered in the

case of *d*-amino acid oxidase. Apparently some enzymes normally resistant become susceptible to oxygen when removed from their cellular milieu.

TABLE IV

*Effect of Preliminary Exposure to 8 Atmospheres of Oxygen upon Subsequent Choline Acetylase Activity of Extracts of Rat Brain*

Rat No.	Preliminary period		Subsequent anaerobic choline acetylase activity; acetylcholine synthesized per gm. original tissue per hr.
		min.	$\gamma$
1	Vacuum, 0°	15	141
	" 37°		98
	O <sub>2</sub> , 1 atmosphere, 37°		39
	" 8 atmospheres, 37°		10
2	Air, 0°	15	142
	Vacuum, 37°		165
	O <sub>2</sub> , 1 atmosphere, 37°		73
3	Vacuum, 37°	60	43
	Air, 37°		56
	O <sub>2</sub> , 8 atmospheres, 37°		15
4	Vacuum, 37°	60	77
	Air, 0°		135
	O <sub>2</sub> , 8 atmospheres, 37°		12

## DISCUSSION

The importance of acetylcholine in the functioning of the nervous system and the unusual prominence of severe symptoms of the central nervous system in acute oxygen poisoning make it tempting to associate the two as cause and effect. We have attempted to make this association by experiments of two types and have been unsuccessful. In the present series of experiments we measured the enzymatic activity concerned with acetylcholine synthesis in the brain by two methods. We could not show that oxygen at high pressure had any effect upon the aerobic synthesis of acetylcholine by brain slices or homogenates. Nor did exposure diminish the choline acetylase activity, as determined by subsequent anaerobic assay, of brain slices exposed to excess oxygen. In a large series of experiments, the results of which are as yet unreported, we attempted to prevent oxygen poisoning in intact rats under 8 atmospheres of oxygen by controlled intraperitoneal injections of acetylcholine. In this we were unsuccessful.

We are unaware of any other experiments in the literature which implicate acetylcholine in the phenomenon of oxygen poisoning.



The curious finding that choline acetylase in extract form is very susceptible to oxygen remains to be explained, together with the parallel finding in the case of *d*-amino acid oxidase.

#### SUMMARY

1. The aerobic synthesis of acetylcholine by slices or homogenates of rat brain is unaffected by exposure to oxygen at high pressure.
2. The choline acetylase activity of slices of rat brain is likewise unimpaired by excess oxygen. However, cell-free preparations of acetylase are rapidly inactivated by oxygen.
3. The relation of these findings to the problem of oxygen poisoning is discussed.

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## ANAEROBIC GLYCOLYSIS IN NERVOUS TISSUE\*

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During the course of a study of the effect of poliomyelitis on the metabolism of nervous tissue, it became necessary to devise a suitable method for the assay of the enzymes of anaerobic glycolysis of nervous tissue. The most feasible measurement seemed to be one conducted in such a way as to give a value for the potential glycolytic rate of tissue. Homogenates offer an opportunity to study tissue in this manner if conditions can be maintained under which enzymes are the limiting factors.

Examination of the literature shows that studies of the rate of anaerobic glycolysis of nervous tissue are mainly limited to reports with brain tissue. The activities of brain homogenates ( $Q_{CO_2}^{N_2}$ , values of 4 to 10) (1, 2) are somewhat inferior to the activities of brain slices ( $Q_{CO_2}^{N_2}$ , values of 15 to 17) (3, 4) and greatly inferior to those of cell-free extracts of brain ( $Q_{CO_2}^{N_2}$ , values of 35 to 50) (3, 5). We have been able to show that the glycolytic activity of homogenates can be made to exceed even that of cell-free extracts if the proper additions of coenzymes and phosphate esters are made. Active cell-free extracts and homogenates have been prepared from cerebrum, cerebellum, medulla, and spinal cord. The activity of these preparations decreased in the order named.

This report is an account of the methods used in the preparation of glycolytic extracts and homogenates from nervous tissue, and a study of the properties of such preparations, especially with a view to their use for assay purposes.

### *Methods*

Hooded rats weighing between 150 and 250 gm. were used in all of these experiments, although the methods have been applied equally well to cotton-rats<sup>1</sup> and in part to mice (6).

The general method of preparation of extracts and homogenates follows: The rats were placed under very light ether anesthesia, decapitated, and the brain and spinal cord were removed. Very little of the peripheral nerve material was included with the cord. In most experiments, the medulla was detached by cutting through the pons and added to the spinal cord. This pooled preparation will be termed "cord" hereafter, while

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<sup>1</sup> Wood, H. G., and Utter, M. F., unpublished experiments.

the remainder of the brain including the cerebellum, cerebrum, and mid-brain will be called "brain." Both preparations may have contained part of the pons, since it was difficult to separate it entirely from either.

After the material was rapidly weighed to an accuracy of approximately 2.5 mg. on a small torsion balance, 4 parts by weight of ice-cold distilled water were added to the tissue and the mixture was homogenized in a Potter and Elvehjem type of grinder (7).

If the tissue was to be used as a homogenate, there was no further treatment. If extracts were to be prepared, the homogenates were incubated for 15 minutes in an ice bath and then centrifuged for 15 minutes in a type SP Sorvall angle centrifuge at approximately 2400 r.p.m. The extracts, especially cord, are not decanted easily and must be removed carefully after centrifugation with a capillary pipette. The centrifugation is sufficient to give a clear extract from brain and a slightly cloudy preparation from cord.

The experiments were carried out for the most part in small Warburg vessels (7 to 9 ml. total capacity) under an atmosphere of 95 per cent  $N_2$  and 5 per cent  $CO_2$  at  $37.5^\circ$ . A typical experiment with all the components necessary for complete activation includes the following substances, expressed as final concentrations, in a total volume of 1.0 ml.: extract or homogenate equivalent to 20 mg. of wet tissue; 0.048 M  $NaHCO_3$ ; 0.01 M  $Na_2HPO_4$ - $KH_2PO_4$  buffer (pH 7.3); 0.028 M glucose; 0.0025 M hexose diphosphate (HDP); 0.0007 M adenosine triphosphate (ATP); 0.0005 M diphosphopyridine nucleotide (DPN); 0.008 M  $MgCl_2$ ; and 0.04 M nicotinamide. Unless otherwise indicated, the tissue was placed in the side arm of the vessel and all other solutions in the main chamber.

In experiments in which chemical analyses were conducted, the contents of the Warburg vessel were diluted with 1.5 ml. of water and aliquots measured out into calibrated test-tubes containing the appropriate deproteinizing agents, which are described below. Phosphate was determined in a trichloroacetic acid filtrate by the method of Fiske and Subbarow (8) as modified by Lohmann and Jendrassik (9). Lactic acid was determined in a  $CdSO_4$ - $NaOH$  filtrate by the colorimetric method of Barker and Summerson (10). Glucose was determined colorimetrically by the method of Folin and Malmros (11) in a tungstic acid filtrate. The determinations were made with the Evelyn or Klett-Summerson photometers.

DPN was prepared from bakers' yeast according to Williamson and Green (12). A biological assay with an apozymase from bakers' yeast (13) indicated a purity of approximately 50 per cent. ATP was prepared from rabbit skeletal muscle by the methods of Kerr (14) and Needham (15). The barium salt on the basis of the 7 minute phosphate was at least 95 per cent pure if calculated as the tetrahydrate. The ratios of

ribose to phosphorus and of 7 minute hydrolyzable phosphorus to total phosphorus equaled the calculated theoretical ratio. HDP was prepared as the sodium salt from the commercial barium salt.

#### EXPERIMENTAL

*Nature of "Inhibitor" of Brain Glycolysis*—Early in this investigation it was realized that, in order to obtain full and sustained activity of nervous tissue preparations, it would be necessary to understand the nature of the "inhibitor" of glycolysis found in brain extracts by Geiger (3, 16) and later by Ochoa (5). Both of these investigators reported that as the proportion of brain extract to total reaction fluid was raised a decreasing rate of glycolysis per unit of brain extract resulted. In order to obtain good activity, the brain extract had to be highly diluted. Ochoa found almost complete inactivity when an extract of 1 part of brain and 4 parts of water constituted approximately 1.0 ml. of the total volume of 1.5 to 2.0 ml. of reaction mixture. This action, if unchecked, would greatly interfere with attempts to use the extract for enzyme assays, since the activity would not be proportional to enzyme concentration.

We have found that this inhibitory action can be controlled if certain precautions are observed in the use of DPN with the brain and cord preparations. It is probable that a DPN nucleotidase, of the type reported by Mann and Quastel (17) and Handler and Klein (18), is responsible for the effect. Both of the above reports showed that added nicotinamide, presumably one of the products of the nucleotidase reaction (18), had a protective action on added DPN. The results presented in Fig. 1 show that nicotinamide has a similar effect with brain and cord extracts. In these experiments, the concentration of DPN was 0.0005 M and the extracts were equivalent to 44 mg. of tissue in a total volume of 2.0 ml. of reaction mixture. The extracts were prepared by homogenizing in 8 parts of distilled water rather than 4 parts, since, as will be shown later, the inhibitory action can be demonstrated at much lower concentrations of extract when 1:8 preparations are used. Curves 1 and 4 respectively show the results with brain and cord extracts when no protection for the DPN from the nucleotidase was provided. The activity fell off rapidly after 10 minutes and ceased entirely in 30 to 40 minutes. At the points indicated, an addition of DPN equal to the initial amount was made from a side arm. This addition caused an immediate increase in the rate which was still evident in the brain extract at 60 minutes, but the rate of the cord extract had again begun to decline at this point. This response to DPN can be interpreted as evidence that lack of DPN was responsible for cessation of activity. As additional evidence, Curves 2 and 5, 0.04 M nicotinamide was added to the reaction mixture to protect the initially added

DPN. An approximately linear rate of gas evolution resulted in both cases and there was no evidence of the action of an inhibitor in either extract.

TABLE I  
*Protection of Diphosphopyridine Nucleotide by Bicarbonate Incubation*

Experiment No.	Components in side arm during preliminary incubation	CO <sub>2</sub> in 60 min. c.mm.
1	Brain extract	162
2	" " , NaHCO <sub>3</sub> , DPN	460
3	" " DPN	19
4	" " NaHCO <sub>3</sub>	64

Brain extract (1:8) equivalent to 44 mg. in a total volume of 2.0 ml. Components as usual except nicotinamide not present. Preliminary incubation period, 23 minutes at 37.5° under air. At the end of this period, 95 per cent N<sub>2</sub> and 5 per cent CO<sub>2</sub> were passed through the flasks for 7 minutes. Total elapsed time before tipping, 33 minutes.

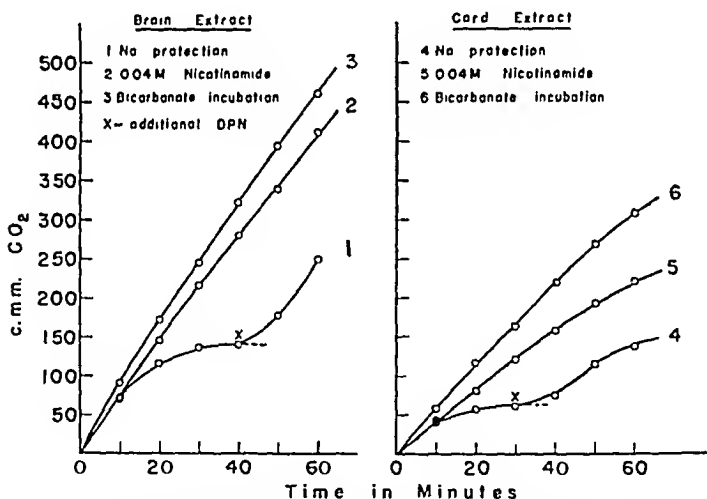


FIG. 1. Protection of diphosphopyridine nucleotide from nucleotidase during glycolysis of brain extract. Addition of DPN equal to the initial amount at X.

A protective measure has been found for DPN which is exclusive of nicotinamide addition and which gives equally good or even better results (Fig. 1, Curves 3 and 6). This method, at present empirical, consists of an incubation of the extract with NaHCO<sub>3</sub> and DPN in the side arm of the Warburg vessel for about 30 minutes prior to addition of the other constituents of the reaction mixture. Table I illustrates the fact that all

three of these components must be present during this incubation period if subsequent protection of the DPN is to result. If the extract is incubated alone in the side arm, activity starts at a normal rate but soon diminishes in a manner similar to Curve 1 of Fig. 1. If the extract is incubated with either  $\text{NaHCO}_3$  or DPN without the other component, almost complete inactivity results. Inclusion of all three components gives linear activity. The incubation protection is successful only if carried out before gas is introduced, apparently because the pH is lowered from above 8 to about 7.5 by the gassing. The presence of oxygen appears to have little effect on the process.

The exact nature of this effect is unknown at present but is under further investigation. Bicarbonate can be replaced by at least one other alkaline

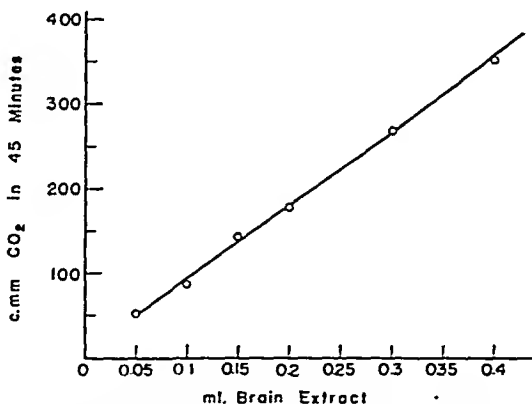


FIG. 2. Change in glycolytic activity with increase in concentration of brain extract

buffer, glycine, but the latter has a different and higher pH range and is generally less effective.

If the nucleotidase action were entirely responsible for the "inhibitor" effect that was reported by Geiger (3) and Ochoa (5), it should be possible to demonstrate a linear relationship between extract concentration and activity when the nucleotidase activity is curbed. This has been done in the experiment of Fig. 2, in which nicotinamide has been used to protect the DPN. A linear relationship exists at least as far as a concentration of 0.4 ml. of brain extract per ml. of reaction mixture. Therefore, we believe that inactivation of the DPN alone is sufficient to account for the inhibitor effect previously observed.

It should be emphasized that the nucleotidase problem is present in

homogenates as well as in extracts and to a greatly intensified extent. Apparently, a large part of the nucleotidase is removed by centrifugation of 1:4 extracts. Nicotinamide exerts a protective effect in homogenate experiments, as will be demonstrated later, but bicarbonate incubation is not successful. In all subsequent experiments, nicotinamide was added to the reaction mixture unless otherwise indicated.

It has been our general impression, although accurate quantitative assays of nucleotidase are not available, that the nucleotidase content of the cord exceeds that of brain. This impression is based on the fact that glycolysis by cord preparations could not be maintained linear as readily as with brain preparation. The glycolysis in both cases was reactivated by addition of DPN.

*Effect of Variation in Preparation of Extracts*—Geiger (3) prepared extracts by grinding the brain in a mortar with 4 parts of either ice-cold dilute

TABLE II  
*Effect of Homogenization Fluid on Activity of Brain Preparations*

Experiment No.	Homogenization fluid	Activity as $Q_{\text{CO}_2}^{\text{N}_2^*}$	
		Extract	Homogenate
1	Distilled water	50.0	46.5
2	Isotonic saline	18.3	31.5
3	0.08 M $\text{NaHCO}_3$	26.8	41.3
4	0.05 " phosphate buffer (pH 5.6)	12.0	32.0

\*  $Q_{\text{CO}_2}^{\text{N}_2}$  = c.mm. of  $\text{CO}_2$  per hour per mg. of dry weight. The brain is assumed to have a dry weight of 20 per cent of the wet tissue. The activity is for the first 60 minute period.

phosphate buffer (pH 5.6) or water, and then centrifuging. We have found that homogenization in the usual type of grinder (7) gives extracts of comparable activity and is a much more convenient process.

The effect of the nature of the homogenization liquid has been investigated (Table II) and it has been found that distilled water gives the best results. In these experiments, two rats were decapitated and the brains were extirpated and the medullas detached. Following this, the brains were halved longitudinally and homogenized as follows: first brain, one-half in distilled water and one-half in isotonic saline; second brain, one-half in 0.05 M phosphate buffer (pH 5.6) and one-half in 0.08 M  $\text{NaHCO}_3$ . In this particular experiment, homogenization in any of the solutions other than distilled water gave extracts with about 50 per cent or less of the activity of the water extracts; the results are somewhat variable, however. The activity of the phosphate extract is slightly at variance with the results

of Geiger who found such extracts about half as active as water extracts. The discrepancy may be due to differences in centrifugation, since the extent of that process is not described in Geiger's report.

The second column of Table II lists the activity of the corresponding homogenates; *i.e.*, uncentrifuged preparations. The activity of the water homogenate (Experiment 1) is lower than that of the water extract, although this is one of a very few cases in which this has been observed. In all cases, however, the activities of the other homogenates are less than that of the water preparation.

The volume of water used in homogenization makes little difference in the activity of extracts if nicotinamide is added to protect the DPN (first column of Table III). However, if nicotinamide is omitted (second column), the volume of extracting liquid becomes important. With a ratio of 1 part of tissue to 4 parts of water, nicotinamide has no effect, indicating that nucleotidase is not present in large quantities. Changing

TABLE III  
*Effect of Volume of Homogenization Fluid on Brain Preparations*

Experiment No.	Ratio, $\frac{\text{brain}}{\text{H}_2\text{O}}$	Activity as $\text{O}_2 \frac{\text{N}_1^*}{\text{CO}_2}$	
		With nicotinamide, 0.04 M	No nicotinamide
1	1:4 extract	31.3	31.6
2	1:8 "	38.9	19.6
3	1:12 "	32.2	12.0
4	1:4 homogenate	50.4	11.2

\* Activity for the first 60 minute period.

the ratio to 1:8, or to 1:12, gives preparations with reduced activity compared to the preparations to which nicotinamide was added. This is an indication that nucleotidase is extracted to a much greater extent with larger volumes of extracting liquid. A corresponding 1:4 homogenate (Experiment 4) shows high activity when nicotinamide is added but is strongly inhibited in its absence. It is, therefore, clear that the nucleotidase is not destroyed by 1:4 homogenization but rather is not extracted. If the homogenization is performed at 1:4 dilution and then additional fluid is added during the extraction period, the same effect is obtained as when the homogenization is done at the final dilution.

It is interesting to note that Handler and Klein (18) believed that the nucleotidase was entirely insoluble in water and saline under their conditions of preparation. This is apparently not true if the cells are disrupted by homogenization in water and if large volumes of extracting fluid are used.



We have found that the period of centrifugation has a considerable effect on the activity of brain extracts (Table IV). Starting with homogenates (no centrifugation), we note a steady decrease in activity as the period of centrifugation is lengthened. The activity of any extract, therefore, is somewhat dependent upon the extent of centrifugation. All of the centrifugations here were performed in a type SP Sorvall angle centrifuge at the indicated speeds.

The inactivation by centrifugation is probably not concerned with nucleotidase, because the preparations were made with 1 part of brain and 4 parts of water, and in addition nicotinamide was included in the reaction mixture. It is probable that the inactivation is caused by removal of hexokinase and adenosinetriphosphatase. At any rate, brain extracts subjected to high speed centrifugation (5 minutes at 18,000 R.P.M. on an International multispeed centrifuge attachment) are no longer active on glucose or HDP. However, addition of arsenate to the extract

TABLE IV  
*Effect of Centrifugation on Activities of Brain Extract*

Experiment No.	Centrifugation	Activity as $Q_{CO_2}^{N_2^*}$
1	None (homogenate)	58.5
2	5 min. (2400 R.P.M.)	42.5
3	15 " (2400 " )	34.8
4	30 " (15 min. at 2400 R.P.M. and 15 min. at 3400 R.P.M.)	22.3

\* Activity of 1:4 brain preparation over the first 60 minute period.

restored partial activity on HDP. The arsenate is believed to avoid the block caused by the accumulation of ATP and the oxidation of triose phosphate can then proceed (19). Normally, this block will not occur if hexokinase is present to cause phosphorylation of glucose or if adenosinetriphosphatase is present (20). Also, it is possible that other enzymes necessary to glycolysis are partly removed by the centrifugation.

*Preparations from Different Sections of Central Nervous System*—In the foregoing experiments, the central nervous system has been divided into two sections labeled "cord" and "brain" and, under this procedure, the medulla was included with the spinal cord. A study of preparations from the individual sections of the brain and cord (Table V) indicates that active extracts and homogenates can be obtained from each section. The combined cerebrum-midbrain and the cerebellum are the most active, the medulla considerably less active, and the spinal cord least active. For this experiment, two animals were sacrificed for each test in order to obtain

sufficient tissue. Extracts and homogenates were prepared in the usual way and tested under optimal conditions. It is noted that if cerebellum is added to the cerebrum-midbrain, it constitutes only about 15 per cent of the total and the activities of the tissues are about equal. In the case of the cord, the medulla constitutes approximately 20 to 25 per cent of the total when added to the spinal cord and probably serves to raise the activity of the preparation slightly.

It is evident from the data of Table V that these methods can be applied to all parts of the central nervous system to obtain extracts and homogenates; all parts glycolyze actively, but at somewhat different rates.

*Necessity of Various Components of Reaction Mixture for Activity*—In the previous experiments, all of the components necessary for maximal

TABLE V  
*Glycolysis of Different Sections of Central Nervous System*

Section	Weight*	Anaerobic glycolysis activity as $\frac{Q}{CO_2} \frac{N_2}{CO_2}$	
		Extract	Homogenate
Spinal cord	gm.		
	1.24	22.5	34.6
Medulla	1.11	31.3	36.5
	0.27	26.5	45.3
Cerebellum	0.41	36.0	48.9
	0.47	39.0	53.3
Cerebrum and midbrain	0.40	46.0	61.9
	2.32	34.8	58.8
	2.25	39.3	64.3

\* Tissues from two rats, pooled.

† Activity for the first 60 minute period.

activity have been added, with the occasional exception of nicotinamide. For full activity of extracts of brain and cord, it is necessary to add the following components, in addition to glucose and buffers: ATP, DPN, and  $MgCl_2$ . For full activity of homogenates and under certain conditions for extracts, it is also necessary to include HDP and nicotinamide.

ATP serves largely as a phosphate carrier. It may also act to a limited extent as a phosphate donor, but HDP serves as the chief reservoir of organic phosphate when such a reservoir is necessary. DPN is necessary as a hydrogen carrier in the oxidative step of glycolysis, and nicotinamide is added to prevent the destruction of DPN. Mg plays a part in several of the glycolytic reactions. For a more complete discussion of the rôle of these components in the glycolysis of brain homogenates see Utter, Reiner, and Wood (6).

The above components are essentially those added in the previous work by Geiger and Ochoa with brain extracts with the exception that Geiger sometimes included phosphocreatine and glutathione. According to Geiger (3), however, the two latter substances gave variable results.

The effect of the omission of the several components is shown for cord extract in Fig. 3 and similarly for brain homogenate in Fig. 4. With two exceptions, the same effects are shown by both preparations.

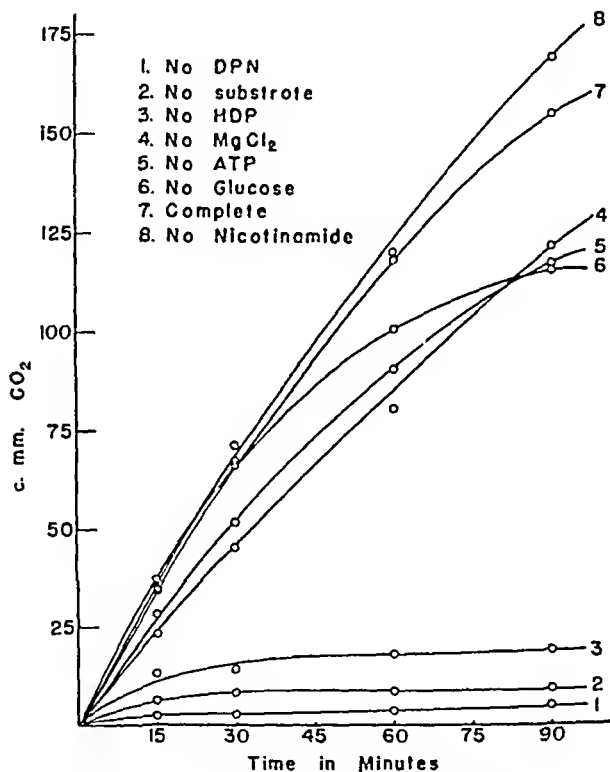


FIG. 3. Necessity of various components for glycolysis of cord extract

Experiments with cord extract (Fig. 3) show that little activity is obtained when either HDP or DPN is omitted (Curves 1 and 3, respectively). The omission of ATP or  $MgCl_2$  (Curves 4 and 5, respectively) has a considerable although smaller effect. If no glucose is added (Curve 6), glycolysis starts at a normal rate but stops when the small amount of initially added HDP (equivalent to 112 c.mm. of  $CO_2$ ) is exhausted.

In the particular experiment of Fig. 3, an amount of 1:4 extract equivalent to 20 mg. of tissue was used in a total volume of 1.0 ml. Under these

conditions, the protective effect of nicotinamide is not necessary. The presence of nicotinamide becomes imperative if a higher concentration of extract is used or if the 1:4 extract is replaced by a 1:8 extract, since in either case the nucleotidase content of the solution is increased.

Actually nicotinamide is slightly inhibitory in the experiment of Fig. 3. This is not unexpected, since nicotinamide may inhibit the oxidative step of glycolysis by competing with DPN for the apoenzyme. Experiments not reported here have shown that the optimal concentration of nicotinamide is about 0.03 to 0.05 *M* and in higher concentration is inhibitory. Handler and Klein (18) reported that the most effective concentration for control of nucleotidase was approximately 0.08 *M*. Thus, nicotinamide apparently plays a dual rôle as an inhibitor and as a protective agent and the most effective concentration will be dependent upon both factors.

Parallel experiments with brain extracts, not shown here, have indicated consistently that similar effects with accessory factors are demonstrable with brain extracts with the exception that the addition of HDP is not always necessary for activity. However, addition of HDP insures far more uniform results. When HDP is omitted, gas output starts at a lower rate than in its presence, although the same or a slightly higher rate is reached after a period of 30 to 45 minutes. The addition of HDP with cord extract is essential as a means of replenishing the organic phosphate which is lost through phosphatase activity. This observation points toward a higher phosphatase activity in cord than in brain, and phosphate determinations (Table VI) support this idea.

The results with brain homogenate (Fig. 4) are similar to those in Fig. 3. The omission of HDP (Curve 1) or DPN (Curve 2) results in extremely limited activity and omission of ATP (Curve 5) or  $Mg^{++}$  (Curve 4) has a smaller but definite effect. In experiments with homogenates, the nicotinamide is essential, as is shown in Curve 3. In the experiments of Fig. 4, the amount of HDP was doubled, giving a final concentration of 0.005 *M*. The HDP was increased since it was found that additional phosphate ester gives a longer constant period of activity. In the absence of glucose (Curve 6), this HDP gives rise to a considerable evolution of gas. However, glucose determinations in Table VI show that glucose is utilized by this preparation even in the presence of HDP. The results with cord homogenates were similar to those with brain.

It is to be noted that in both Figs. 3 and 4 the completely supplemented preparations exhibit approximately linear activity with time and they therefore appear suitable, at least in this respect, for assay purposes.

Previous experiments with mouse brain homogenates (6) have indicated that nicotinamide and ATP play less important rôles in the mouse preparations.

*Chemical Determination of Products*—Although the production of acid as followed manometrically is usually considered to give a reasonably accurate measure of glyeolytic activity, additional and more exact information can be obtained when chemical determinations supplement the manometric data. In the experiments of Table VI, glucose, lactate, and phosphate changes were measured in addition to the usual  $\text{CO}_2$  production. The values are given as mm per gm. of wet tissue per hour, and the various preparations can be compared directly.

It should be pointed out that the glucose values as determined are probably slightly in error, since the determinations by the Folin and

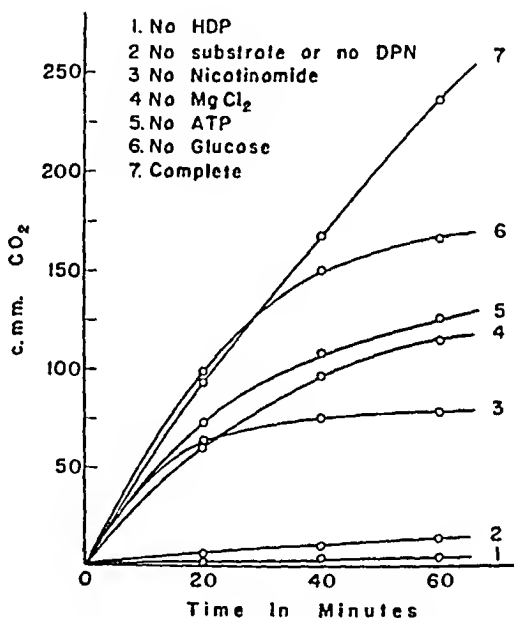


FIG. 4. Necessity of various components for glycolysis of brain homogenate

Malmros method (11) are subject to some error in the presence of phosphate esters. According to LePage and Umbreit (21), under the conditions of this method HDP has a reducing value of 9.5 per cent of that of glucose, glucose-6-phosphate 13.2 per cent, and fructose-6-phosphate 31.7 per cent. On this basis, the correction for the original amount of HDP would be quite small, 0.9 per cent of the glucose in Experiments 2 and 4 (Table VI), and 1.8 per cent in Experiments 1 and 3. However, considerable inorganic phosphate was esterified during the experiments with brain extracts (Experiments 4 and 5) and hexose diphosphate and hexose monophosphate are undoubtedly formed. Since no accurate determinations

of these compounds were made, no correction could be applied to the glucose values. The net effect of the error probably is to decrease the value obtained for the glucose utilized in Experiments 4 and 5.

The determinations of Table VI were carried out on a substrate of glucose and HDP and with four preparations: (Experiment 1) cord homogenate, (Experiment 2) cord extract, (Experiment 3) brain homogenate, and (Experiment 4) brain extract. In addition, brain extract was tested on a substrate of glucose alone (Experiment 5).

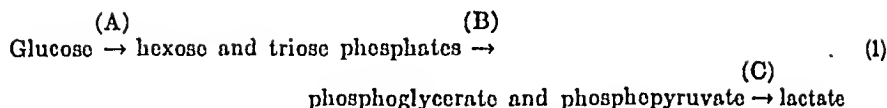
Chiefly answers to two questions were desired: (a) Is  $\text{CO}_2$  production from bicarbonate a reliable indication of lactic acid production, and (b) what changes in inorganic phosphate and phosphate esters occur during glycolysis in the various preparations?

TABLE VI  
*Chemical Determinations of Glycolysis of Nervous Tissue*

Ex- per- iment No.	Preparation	Substrate	mm per gm. brain per hr.				Ratio, $\frac{\text{glucose}}{\text{lactate}}$
			$\text{CO}_2$ from NaHCO <sub>3</sub>	Lactic acid pro- duced	Glucose utilized	Inorganic P change	
1	Cord homogenate	0.014 M glucose	0.330	0.297	0.060	+0.137	0.20
2	" extract	0.0025 " HDP					
		0.014 " glucose	0.181	0.178	0.091	-0.016	0.51
		0.00125 M HDP					
3	Brain homogenate	0.014 M glucose	0.523	0.478	0.175	+0.198	0.37
		0.0025 " HDP					
4	" extract	0.014 " glucose	0.389	0.368	0.222	-0.152	0.60
		0.00125 M HDP					
5	" "	0.028 M glucose	0.317	0.343	0.179	-0.123	0.52

\* Hexose diphosphate.

A cursory examination of Table VI shows that the  $\text{CO}_2$  and lactate values check reasonably well in all cases. This agreement indicates that it is fairly safe to use manometric figures as a rough measure of lactic acid. However, we believe that the agreement between the  $\text{CO}_2$  and lactic acid columns is partly fortuitous since the  $\text{CO}_2$  values are subject to at least three sources of error: (1) retention of  $\text{CO}_2$  in the reaction mixture, (2) changes in acidity due to esterification of phosphate or hydrolysis of phosphate esters (*cf.* Ochoa (5)), and (3) formation of phosphoglyceric or phosphopyruvic acids from HDP. The formation of the latter acids will result in the release of  $\text{CO}_2$  but will not contribute to the lactic acid. The error mentioned in (3) will occur if Reaction B is more rapid than Reaction C in Equation 1. This type of reaction may occur when HDP is added to the reaction mixture.



We have not made any corrections on the figures of Table VI. However, if the single correction (2) for phosphate ester changes is made in Experiments 4 and 5, the results agree with similar experiments by Ochoa (5) who made this type of correction.

If glucose is quantitatively converted to lactic acid and neither compound is involved in other reactions either as a product or as a reactant, the ratio of glucose utilized to lactic acid produced should be 0.5. Actually, the situation is more complicated, since the intermediate changes of hexose and triose phosphates, etc., occurring during glycolysis will influence the ratio. For example, in Equation 1, with a substrate of glucose and HDP, if the rates of Reactions B and C exceed that of Reaction A, the concentration of hexose phosphates will decrease during the reaction and a glucose to lactate ratio of less than 0.5 will result. On the other hand, if Reaction A exceeds Reactions B and C in velocity, the ratio will be greater than 0.5 since phosphate esters will accumulate.

An examination of the "Glucose utilized" column of Table VI shows that glucose disappears rather slowly during the glycolysis by homogenates, especially in the case of the cord preparation. This slow utilization of glucose is reflected in a lowered glucose to lactate ratio (last column) and indicates that, in homogenates, the speed of Reactions B and C exceeds that of Reaction A. This is confirmed by the inorganic phosphate figures, since a net increase in phosphate indicates a net decrease in phosphate esters during the reaction. If the rates of Reactions A, B, and C are well balanced, there should be no change in inorganic phosphate and the glucose to lactate ratio should be 0.5. This is the situation with cord extract (Experiment 2), although different samples of cord extract show some variation in this respect. Brain extracts exhibit a different picture. Here, inorganic phosphate is taken up, pointing to a rate in Reaction A more rapid than that of Reactions B and C. The ratio of glucose to lactate is above 0.5, as is to be expected when part of the glucose is converted to esters. As explained earlier, the determined values for the glucose utilized are probably too low and the expressed values for glucose to lactate ratios are minimal figures.

*Effect of Ether upon Glycolysis*—It is a distinct advantage in handling some animals, particularly cotton-rats, to precede decapitation with anesthetization. A series of experiments, therefore, was carried out (Table VII) to determine whether anesthetization with ether has any effect on the activity of homogenates and extracts prepared from the

nervous tissue. In the ether treatment, the animals were placed in a closed box fitted with a window which permitted observation. When under light anesthesia, the animal was removed and decapitated. In the control group, there was no previous treatment. Extracts and homogenates were prepared from brain and cord as described previously and the anaerobic glycolysis was determined manometrically for 1 hour under optimal conditions. Duplicate determinations were made on each preparation; *i.e.*, the twelve determinations on cord extract represent duplicate experiments on six animals.

The data indicate that previous etherization has no effect on extracts of either cord or brain and may cause a small increase or none in the activity of homogenates, especially in the case of cord. It is probable that ether may be removed by the exposure to the air during the treatment of the

TABLE VII

*Effect of Ether upon Glycolysis of Nervous Tissue*

Values as  $Q_{CO_2}^{N_2}$  for the first 60 minute period.

	Cord extract		Cord homogenate		Brain extract		Brain homogenate	
	Ether	No ether	Ether	No ether	Ether	No ether	Ether	No ether
Number of determinations	12	12	12	12	10	10	9	9
Range	16.0-26.0	17.5-25.5	29.8-46.0	27.0-40.8	23.3-49.8	25.5-53.8	48.0-61.5	50.5-58.0
Mean	21.5	21.6	39.6	35.7	37.7	37.5	55.7	53.8
Difference between means	0.1		3.8		0.2		1.9	
Standard error of difference between means	1.26		1.95		3.86		1.79	

tissue and by the passage of gas through the manometer vessel during the gassing period. The larger solid content of homogenates may tend to retain the ether to a greater extent than with the extracts. Treated statistically, for the standard error of the difference between means, the difference is probably significant in the case of the cord homogenate but is not significant in the case of the brain homogenate. However, this particular type of analysis may be misleading, since all factors, including a considerable variation between different runs in which preparations from both etherized and normal animals were affected similarly, are lumped together with other variations.

Craig (22) reported that slices of cat cerebral cortex showed a slightly increased lactic acid production under aerobic conditions when ether was added directly to the manometer cup. This was true even in the case of



very small concentrations of ether. This observation is in agreement with the above results with brain and cord homogenates. Beecher and Craig (23) also suggested that lactic acid output from cerebral cortex slices was greater when the tissue was obtained from cats under ether anesthesia if measurements were conducted in a phosphate medium. On the other hand, Craig (22) and Jowett and Quastel (24) conclude that ether in sublethal doses has little effect on the oxidative processes of brain as determined *in vitro*.

The results of Table VII also give an idea of the range and average activity of the various preparations. However, following this experiment, one source of variation between runs was discovered and corrected. Nicotinamide varies in its effect somewhat with the time since preparation, and more consistent results were obtained when a fresh solution was prepared each day.

*Use of Extracts and Homogenates for Assay of Glycolytic Enzymes*—After determination of the conditions necessary for optimal activity, it was possible to examine completely activated preparations with regard to their suitability for assay of the glycolytic enzymes. In order to be useful for assay purposes it has generally been considered that the preparations must at least fulfil two requirements: (a) exhibit a linear relationship between enzyme concentration and activity, *i.e.*, the concentration of enzymes must be the limiting factor in the reaction, and (b) the preparations must exhibit a constant activity for a reasonable period of time.

We believe that both the homogenates and extracts meet these specifications reasonably well if certain definite precautions are observed. In Fig. 5 it is demonstrated that a linear relationship exists between the amount of cord and brain homogenates and the activity. This linearity exists (a) if no more than 0.1 ml. of a 1:4 homogenate is used in a total volume of 1.0 ml., (b) if the period of activity is limited to 60 minutes, and (c) if the initial DPN concentration is raised to 0.001 M.

In the right half of Fig. 5, a similar experiment with cord extract is shown in which the amount of extract was increased to 0.3 ml. in a total volume of 1.0 ml. It is evident that the activity is fairly linear up to a concentration of 0.1 ml. and probably to 0.2 ml. of a 1:4 extract for a 60 minute period and other experiments have indicated that this period can be lengthened to 90 minutes. In this test DPN and HDP were the usual 0.0005 M and 0.0025 M concentrations, respectively.

A similar experiment with brain extract has been shown in Fig. 2. The situation is considerably more favorable here, since the amount of brain extract used may be quite high.

The assumption that enzyme concentration is limiting, when linearity is obtained with increasing concentration of enzyme, is open to at least

one possible error. If an unknown, non-enzymatic cofactor capable of limiting the activity is present in the enzyme preparation, addition of an increasing amount of the enzyme preparation may give a linear relationship because of the cofactor rather than the enzymes. We have made an attempt to test this possibility. If such a cofactor is limiting activity and is heat-stable, it should be possible to destroy the glycolytic enzymes by a short heating period and leave the cofactor intact. Addition of the heated preparation should then increase the activity of an unheated preparation.

In the experiments of Table VIII, up to 3 volumes of heated extracts from brain and cord were added to unheated extract and the effect upon

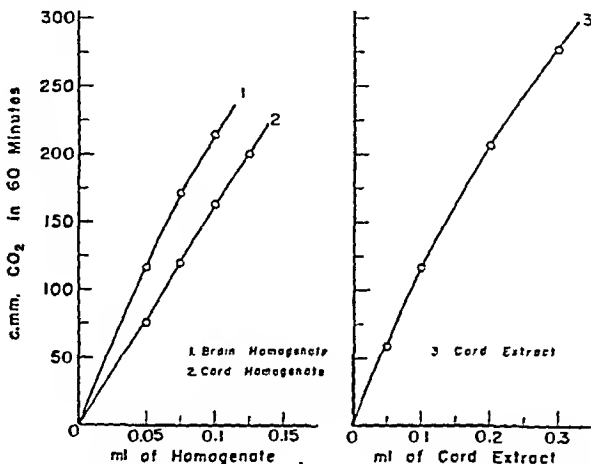


FIG. 5. Changes in glycolytic activity with increased concentrations of brain and cord preparations.

the activity was noted. An increase in activity of about 25 per cent occurred in the case of the cord and a slightly smaller increase with brain. It is probable that the increase occurs because the conditions of glycolysis are not quite optimal with regard to phosphate esters, inorganic ions, etc., rather than because an unknown cofactor is limiting. As mentioned previously, Geiger (3) got small and irregular increases in the activity of brain extract by adding glutathione and phosphocreatine, and the situation might be comparable here. At any rate, if there is a cofactor in the extract, it is improbable that it is sufficiently limiting to cause a serious error in glycolytic assays. This experiment does not eliminate the possibility of a heat-labile factor, of course.

Concerning the second point, constancy of activity, a glance at previous experiments will show that the preparations are reasonably satisfactory in this respect. In Fig. 1 brain and cord extracts are quite constant in activity if proper precautions are observed. Although completely activated brain homogenate (Fig. 4, Curve 7) shows some decrease in activity with successive readings, it is fairly linear. Cord homogenate is similar to brain homogenate in this respect.

Certain departures from a constant rate of activity are to be expected in any complicated assay system. Previously, some of the interrelationships of different phases of glycolysis have been discussed. Changes in the proportions of the various intermediates (Equation 1) during the course of the assay may have an effect on gas evolution and hence upon the constancy of the rate. For example, at the start of a reaction, HDP may

TABLE VIII  
*Effect of Heated Extracts on Glycolysis by Brain and Cord*

Experiment No.	Heated extract added*	Activity as $\frac{O}{CO_2}$	
		Cord	Brain
	ml.		
1	0.0	22.5	32.2
2	0.1	25.4	35.7
3	0.2	27.5	35.0
4	0.3	27.9	36.9

\* Heated extract prepared by boiling 1:4 extract for 10 minutes and centrifuging. Each flask contained 0.1 ml. of unheated cord or brain extract (1:4) and the indicated amount of corresponding heated extract in a total volume of 1.15 ml.

be converted to phosphoglyceric acid very rapidly, with a consequent evolution of  $CO_2$ . Later in the reaction, when the concentration of HDP is reduced, the rate of formation of phosphoglyceric acid may decrease and  $CO_2$  evolution will decrease likewise.

*Keeping Properties of Preparation*—Although no extensive investigation has been made of the keeping properties of the various preparations, a few experiments have given some evidence bearing upon this question. Freezing slowly by placing in the freezing tray of a refrigerator caused some loss in activity. The loss, usually less than 30 per cent, apparently occurred during the freezing process and the subsequent loss in activity following the initial freezing was rather slow (activity down to approximately 50 per cent in 5 days). If the preparations were placed in sealed vials and frozen rapidly in a dry ice-acetone mixture and were then kept at dry ice temperatures, there was little change in activity, at least over a period of 4 to 5 days.

Lyophilizing yielded preparations which retained their original activity approximately, but drying of unfrozen preparations caused considerable loss of activity.

#### DISCUSSION

The results of these experiments with preparations of nervous tissue indicate that anaerobic glycolysis takes place in such preparations by means of the usually accepted cycle of phosphorylated intermediates. These observations confirm the work of Ochoa (5), Meyerhof and Perdigon (25), and McFarlane and Weil-Malherbe (4) but differ from earlier reports of a non-phosphorylating glycolytic path for brain tissue (26, 27).

Geiger (3) reported brain extracts unable to form lactic acid from hexose diphosphate or from hexose monophosphate. Ochoa (5), however, found that brain extracts glycolyzed readily at the expense of these two esters. Our results confirm Ochoa's report that lactic acid can be formed from HDP even in the absence of any added phosphate acceptor.

The present work points to similar glycolytic paths in brain and cord, with variations quantitative rather than qualitative in nature.

Huszk (28) found that "gray" matter from cat or ox brain was able to phosphorylate glucose by means of ATP and also could initiate glycolysis by phosphorylation of glycogen. "White" matter from brain, on the other hand, was reported to be unable to phosphorylate glucose and it was suggested that "white" matter glycolyzed chiefly by utilizing glycogen or phosphorylated intermediates. The results reported here do not apply directly to this question, since both brain and cord are mixed preparations containing both types of nervous tissue. However, it seems possible to advance an alternative explanation for Huszk's failure to find phosphorylation of glucose with "white" material which could be based on a possible higher phosphatase activity of preparations from "white" matter compared with preparations from "gray" matter. The activity of phosphatase might be so much greater than the hexokinase activity that little phosphorylation of glucose could occur. For example, Ochoa (5) prepared an acetone powder from brain which contained hexokinase and could phosphorylate glucose at the expense of ATP. However, the hexokinase reaction could be detected only if the phosphatase reaction was inhibited by added NaF. The fact that phosphorylation of glucose could not be detected by Huszk even when hexokinase from "gray" matter was added may indicate that it was not hexokinase but the ATP which was the missing component of the reaction.

It should perhaps be emphasized that the type of assay which is attained by use of preparations similar to those described in this report is limited to an attempt to determine the entire potential activity of an enzyme system

in cells which have been disrupted as completely as possible. It is obvious that this does not enable one to predict, without further information, the actual activity *in vivo* of the enzymes, which may be limited by concentrations of substrate and coenzymes and by the restrictions imposed by cellular structure. However, a knowledge of the maximal activity of the enzyme is an indispensable part of the information which will be required for making such predictions from measurements *in vitro*.

In an assay of the entire glycolytic system, the rate will be determined approximately by the slowest enzyme or enzymes, and consequently little information concerning concentration of other enzymes may be divulged. Nevertheless, an over-all assay, if conducted in conjunction with sufficient chemical determinations, is a good method of learning the internal relationships of various parts of the entire enzyme system, and particularly of detecting the slower enzymes which then can be assayed more specifically.

It is interesting to compare brain homogenates, fully fortified, as described in this report, with somewhat similar preparations as obtained by Elliott and Henry (29). These investigators obtained suspensions from brain by grinding in a Ringer-bicarbonate-saline solution and observed activities approximating those of brain slices; the activity of slices (3, 4) is about one-third to one-fourth the activity of our homogenates. No additions, other than buffers and substrate and occasionally Mg and pyruvate, were mentioned and the activity was actually increased by washing the suspensions. It seems probable that these preparations contained a much larger proportion of unbroken cells and larger cell fragments than is the case with our distilled water homogenates. Elliott and Henry's suspensions are probably not as suitable for measurement of total potential activity as are the present preparations, but on the other hand, may approximate conditions *in vivo* and be very useful from this point of view.

The authors gratefully acknowledge the technical assistance of Miss Carol Carlson and Miss Ollie Stubblefield.

#### SUMMARY

1. A method is described for the preparation from the central nervous system of cell-free extracts and homogenates exhibiting high rates of anaerobic glycolysis. A study of the variable factors in the preparation of the extracts and homogenates has been made.

2. Active extracts and homogenates have been prepared from spinal cord, medulla, cerebellum, and cerebrum.

3. For complete activity these preparations need, in addition to glucose and buffers, adenosine triphosphate, hexose diphosphate, diphosphopyridine nucleotide, magnesium ions, and nicotinamide.

4. Evidence is presented that the "inhibitor" of brain extract reported by Geiger (3, 16) is a diphosphopyridine nucleotidase. Inhibition of the nucleotidase can be obtained with nicotinamide or by incubation of the preparation with DPN and bicarbonate buffer.

5. Etherization of animals prior to preparation of glycolytic extracts and homogenates did not have any detectable effect on extracts and had slight or no effect on homogenates.

6. The preparations have approximately linear activity with time and with increasing concentration of tissue for 60 minutes or longer. They, therefore, should prove useful in the assay of enzymes of anaerobic glycolysis of the central nervous system.

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# THE RÔLE OF MALTASE IN THE ENZYMOLYSIS OF RAW STARCH\*

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In the preceding publication on the digestion of raw starch (1), it was reported that granules of wheat, corn, or potato starch can be rapidly and completely converted to fermentable sugars by a mixture of enzymes extracted from hog pancreas and *Aspergillus oryzae* grown on bran. Glucose is the preponderant end-product. The difference between the hydrolysis of raw and cooked starch appears to be one of rate; the conversion of boiled starch to sugars by this mixture of enzymes is more rapid and equally complete. This completeness of hydrolysis seems more remarkable than its occurrence with raw starch.

Microscopic observation of wheat or corn-starch during disintegration indicated that the enzymic attack did not occur initially over the surface of the granule, but proceeded radially from the hilum, where most of the moisture of the original granule is concentrated (2).

The mixture of enzymes used in the preceding work was too complicated to permit an explanation of their mode of action; yet knowledge on this point should be important to an understanding of amylases and the structure of starch. The present paper presents observations made with greatly purified enzyme preparations.

Pancreas and *Aspergillus* are both known to contain much  $\alpha$ -amylase, but the action of  $\alpha$ -amylase alone on uncooked starch, except in the presence of extremely high concentrations of enzyme, reaches completion very slowly.  $\beta$ -Amylase on the other hand has been shown by other experimenters (3, 4) to have no effect on raw starch. The observation has been repeated by us with  $\beta$ -amylase that acted as a single protein in the ultracentrifuge. It was found to have no action alone on raw starch, and no apparent effect on the action of the pancreas-mold mixture. It is obvious, therefore, that some factor in addition to the recognized amylolytic ferments is involved in the rapid digestion of raw starch.

Certain recorded observations suggest that this factor may be an  $\alpha$ -glucosidase such as maltase. Thus Stark (5) has demonstrated a "pseudo-

\* Enzyme Research Laboratory Contribution No. 96. This work was done under the special research fund authorized by the Bankhead-Jones Act of June 29, 1935.



equilibrium" between dextrans and maltose, and Pigman (6) has shown that maltose can be converted into non-fermentable products in the presence of amylases. Obviously these effects would be hindered by maltase.

By using a preparation (from pancreas) containing  $\alpha$ -amylase but practically no maltase, and a preparation (from *Aspergillus*) containing much maltase but no  $\alpha$ -amylase, it has been found that the rapid and complete digestion of raw starch occurs because of the removal of maltose. This statement holds despite the possibility that other enzymes not here taken into account may exist in the partly purified preparation from pancreas and *Aspergillus*, because similarly rapid digestion of raw starch has been made with the  $\alpha$ -amylase preparation alone, but only when the maltose was continuously removed by dialysis.

#### EXPERIMENTAL

##### *Determination of Enzyme Activity*

*$\alpha$ -Dextrinogenic Activity*—This term (3) has been used to denote the activity of  $\alpha$ -amylase in the presence of excess  $\beta$ -amylase. The determination is a modification of the procedure of Sandstedt, Kneen, and Blish (7). A starch fully acted upon by  $\beta$ -amylase still gives a purple color with iodine. Subsequent action of  $\alpha$ -amylase destroys this property. The rate of destruction of the chromogenic property is a function of the concentration of  $\alpha$ -amylase.

A strong solution of  $\beta$ -amylase is prepared by stirring 40 gm. of whole hard wheat meal into 100 ml. of water at room temperature and removing the insoluble portion in a centrifuge. 2 ml. of this solution are incubated at 30° for 10 minutes with 20 ml. of a 2 per cent suspension of soluble starch in 0.02 M acetate, pH 4.8. Immediately thereafter 8 ml. of the solution to be tested are added. (This solution should contain about 0.1 per cent of calcium chloride.) Incubation at 30° is continued, and at suitable intervals (usually every 1 or 2 minutes) 0.5 ml. portions of the mixture are removed and mixed with 5 ml. of an iodine solution containing 0.088 gm. of iodine and 40 gm. of potassium iodide per liter. The intensity of the resulting color is determined at once. The time that would be required to produce a color arbitrarily selected as a standard is found by interpolation.<sup>1</sup> Under

<sup>1</sup> Sandstedt, Kneen, and Blish (7) develop a color in similar fashion and compare it visually with a standard tube containing iodine and a definite amount of "reagent" dextrin. In the present experiments the standard used was a reading of 40 in a neutral wedge (visual) colorimeter with a quarter inch cell and a 540 m $\mu$  filter. Unfortunately it is difficult to describe this color in terms of another instrument. As the actual units are only a matter of convenience, it should be possible to define a suitable unit for another colorimeter. The units for  $\alpha$ -amylase (per mg.) used here represent about twice as much enzyme activity as those of Sandstedt, Kneen, and Blish.

the conditions described, the time corresponds to a certain quantity of  $\alpha$ -amylase. 1  $\alpha$ -amylase unit is defined as that amount of enzyme in the total digestion mixture of 30 ml. that will produce the standard color in 10 minutes. A curve (Fig. 1) shows the relationship between the time required to reach the standard color and various concentrations of a preparation of salivary amylase.

*Saccharogenic Activity*—The saccharogenic activity is reported in terms of sugar formed. 1 unit of activity has been defined as that amount of enzyme that converts 1 ml. of a 2 per cent cooked, wheat starch suspension (pH 5.2) at 45° to fermentable sugars at an initial rate of 10 per cent per minute.

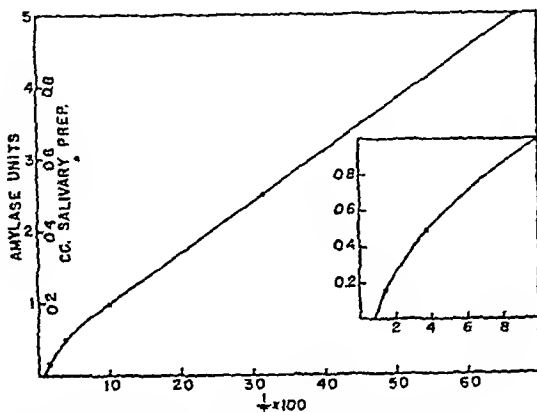


FIG. 1. Showing the relationship between  $\alpha$ -amylase units and the time ( $T$ ) required to reach standard color. The inset is on a larger scale.

The sugars were determined as previously reported (1) by a combination of alkaline ferricyanide and acid copper reduction methods, except that the enzymic reaction was stopped by the addition of 1 ml. of 1  $N$  HCl per ml. of digestion mixture instead of with sodium hydroxide. The system was then diluted and neutralized with  $Na_2CO_3$  to pH 4.0 for fermentation or to pH 7.0 for measurement of the total reducing capacity. It was found convenient at times to replace the ferricyanide method with Nelson's colorimeter adaptation (8) of the Somogyi procedure (9). The undigested starch which Nelson found interfered with the clarity of the final colored solution could be removed by allowing the retrograded starch to settle in the cold. Determined in this way the ratio of saccharifying to  $\alpha$ -amylase activity was found to be different for different sources of  $\alpha$ -amylase practically devoid of  $\beta$ -amylase. Thus, in Table I the ratio is 2.8 for the amylase

of pancreas, whereas the ratio is 1.7 for salivary amylase and 1.1 for *Aspergillus* amylase.

**Maltase**—5 ml. of a 1 per cent solution of maltose, containing 0.1 M acetate buffer, pH 5.2, were incubated at 40° with 5 ml. of enzyme sample. At the end of 30 minutes, 1 ml. of the digestion mixture was pipetted into 1 ml. of 1 N HCl. The mixture was neutralized with Na<sub>2</sub>CO<sub>3</sub>, and the glu-

TABLE I  
*Enzymic Activities of Various Preparations*

Designation	Source of preparation	Enzyme activities		
		$\alpha$ -Amylase	Saccharogenic	Maltase
		units per mg.	units per mg.	units per mg.
$\alpha$ -Amylase I	Pancreas solids	0.4		
“ II	“ extract	2.3	6.4	$0.08 \times 10^{-1}$
“ III	Salivary solids	4.0	7.0	0.00
Taka-diastrase I.	<i>Aspergillus oryzae</i> , extract	$5.5 \times 10^{-1}$		0.06
“ II.	<i>Aspergillus oryzae</i> , alcohol fraction	$0.5 \times 10^{-1}$		$0.08 \times 10^{-1}$
“ III.	<i>Aspergillus oryzae</i> , alcohol fraction	0.4		0.19
Mold Bran I. . . .	<i>Aspergillus oryzae</i> , commercial prepara- tion	$1.1 \times 10^{-1}$		$0.03 \times 10^{-1}$
“ “ II	<i>Aspergillus oryzae</i> , solids	5.3	5.9	0.15
Maltase I ..	<i>Aspergillus oryzae</i> , from mold bran	$0.1 \times 10^{-1}$	$0.2 \times 10^{-2}$	0.16
“ II . . .	<i>Aspergillus oryzae</i> , from mold bran	$0.5 \times 10^{-3}$		0.24
“ III ...	<i>Aspergillus oryzae</i> , from taka-diastrase	$0.2 \times 10^{-1}$		0.59
“ IV	<i>Aspergillus oryzae</i> , from mold bran	$0.8 \times 10^{-1}$		0.49
$\beta$ -Amylase I..	Sweet potato, purified	$0.3 \times 10^{-3}$	3.2	0.00
“ II..	Wheat, extracted with water	$0.1 \times 10^{-3}$	$1.2 \times 10^{-1}$	

cose present was determined as described previously. 1 unit of maltase has been defined as that amount of enzyme in the total digestion mixture that hydrolyzes 10 per cent of the maltose under these conditions.

**Digestion of Raw Starch**—Unless otherwise stated, the procedure was as follows: The substrate was a washed air-dried sample of commercial wheat starch (free of protein) whose carbohydrate content when determined by hydrolysis with HCl amounted to 88.5 per cent. 22 mg. of this starch were

weighed into a 3 ml. round bottomed flask. Into this flask were pipetted such amounts of material that the final volume of the suspension was 1 ml. The concentrations or quantities of reagents were acetate buffer (pH 5.2) 0.05 M,  $\text{CaCl}_2$  0.05 per cent,  $\alpha$ -amylase (No. II) 0.5 mg. (equivalent to 1.15 units), and Maltase I 2 mg. (equivalent to 0.32 unit). Conversion was carried out in a water bath at 45°. Continuous stirring was provided by placing the flask in a test-tube attached to a pulley by a joint slightly out of the perpendicular, so that the test-tube swung somewhat with each revolution of the pulley. The reaction was stopped by the addition of 1 ml. of 1 N HCl.

When it was desired to compare cooked starch with raw starch as substrate, the same procedure was used, but the flask containing the starch in 0.5 ml. of  $\text{H}_2\text{O}$  was stirred in a bath of boiling water for 30 minutes before use.

### *Enzyme Preparations*

*$\alpha$ -Amylase*—Table I lists the enzymic activity of amylase preparations from various sources.  $\alpha$ -Amylase I was a sample of commercial pancreatin.  $\alpha$ -Amylase II was prepared from a commercial "amylpsin" by drying the water-soluble material remaining after dialysis.  $\alpha$ -Amylase III was prepared by drying human saliva, after prior purification by centrifugation, filtration, dialysis, and precipitation in 70 per cent alcohol.

*Maltase Preparations*—The stability of taka-maltase toward acid pH, high temperature (10, 11), and aqueous alcohol (11, 12) is in marked contrast with the behavior of yeast maltase (13). These properties have been used in the preparation of an  $\alpha$ -amylase-free maltase preparation from taka-diastrase. The use of acid and precipitation by alcohol was particularly effective. In Table I, the taka-diastrase preparations represent, respectively, (No. I) the original commercial sample, (No. II) a fraction thereof obtained by dialyzing a 10 per cent solution, then precipitating in 50 per cent alcohol, and (No. III) the residue obtained from the foregoing alcoholic mother liquor by increasing the concentration of alcohol to 70 per cent.

Maltases I and II were prepared from commercial mold bran. 100 gm. of mold bran were extracted for 1 hour at room temperature with 1 liter of water. The extract was squeezed through cheese-cloth and then centrifuged. The resulting supernatant liquid yielded a clear brown solution after filtration through diatomaceous earth. Ammonium sulfate was then added to complete saturation. (It was found that the yield of maltase in the precipitate increased with increasing  $(\text{NH}_4)_2\text{SO}_4$  concentration to complete saturation.) The precipitate was filtered out on purified diatomaceous earth, dissolved in 100 ml. of  $\text{H}_2\text{O}$ , and brought to pH 3.0 with

1 N HCl. The presence of considerable sulfate decreased the concentration of calcium ion, thus contributing to the lability of the  $\alpha$ -amylase. After remaining at 30° for 30 minutes, the preparation was cooled to 0° and precipitated with cold 95 per cent ethyl alcohol at a final concentration of 70 per cent. The resulting precipitate was dialyzed overnight in a double walled cellophane membrane against tap water and then against distilled water. (Considerable cellulase is present and makes the use of a single walled cellophane membrane somewhat risky.) The insoluble matter remaining after dialysis was removed by filtration and the resulting filtrate was dried.

Maltase III was made from Taka-diastrase III. The latter preparation was suspended in water, fractionated with alcohol as before, and the resulting precipitate with 70 per cent alcohol was again suspended in water and adjusted to pH 3. The suspension was kept at 30° for 1 hour, then dialyzed, filtered, and dried.

Maltase IV was prepared as was Maltase I except that the original mold bran extract was acidified and neutralized again before precipitation with ammonium sulfate.

$\beta$ -Amylase— $\beta$ -Amylase I was a highly purified preparation from sweet potatoes, which sedimented as a single protein in the ultracentrifuge.<sup>2</sup>  $\beta$ -Amylase II was a dried, water extract of ground whole wheat.

All preparations were dried *in vacuo* while frozen.

### Results

*Course of the Over-All Conversion*—Relatively large concentrations (1.15 units per ml.) of  $\alpha$ -amylase alone were found to digest both raw and cooked starch to the extent of about 65 per cent in 5 hours at 45° (Fig. 2), although at considerably different initial rates (Figs. 3 and 4). Glucose was formed from both raw and cooked starch, as reported by Stark (5) and by Myrbäck and Lundberg (14). However, more glucose was formed from raw than from cooked starch.

Fig. 2 also illustrates a sharp change at about 45 per cent in the course of the hydrolysis of cooked starch, again in agreement with the findings of Myrbäck (15). The change in rate was less marked when added maltase was present. A similar change in the rate of hydrolysis of raw starch was also far less marked. The maximum rate of hydrolysis of cooked starch by  $\alpha$ -amylase, with or without maltase, is the initial rate, but, as shown in Fig. 3, there appears to be a lag in the formation of sugar from raw starch. In the presence of maltase this initial lag did not occur.

*Effect of Varying Concentration of  $\alpha$ -Amylase*—With raw starch, increase in the concentration of  $\alpha$ -amylase resulted in a higher total conversion as

<sup>2</sup> Unpublished results of A. K. Balls and R. R. Thompson.

well as a higher rate of conversion (Table II). However, the amount of glucose ultimately formed was independent of the concentration of  $\alpha$ -amylase. Enzyme preparations of salivary and pancreas origin were indistinguishable in these respects.

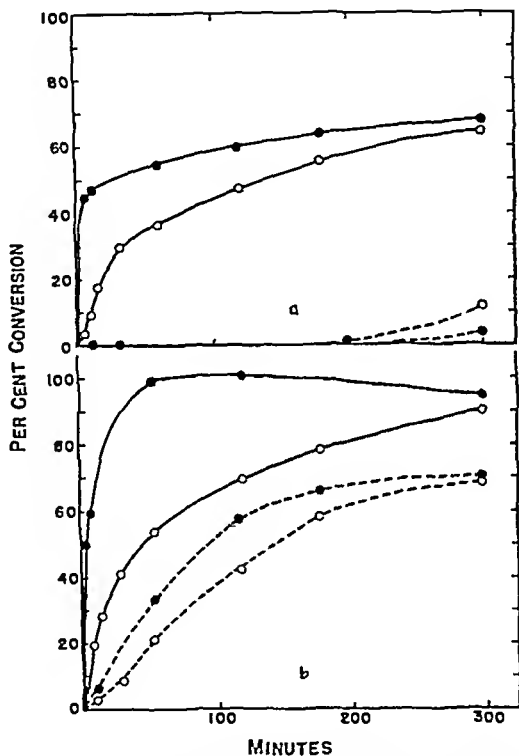


FIG. 2. Course and extent of hydrolysis of raw starch (open circles) and cooked starch (solid circles) by (a)  $\alpha$ -amylase and (b)  $\alpha$ -amylase plus maltase. The continuous curves represent the percentage of fermentable sugars, whereas the broken curves represent the percentage of glucose.

The maximum velocity of hydrolysis attained at different  $\alpha$ -amylase concentrations is shown in Fig. 4 for both cooked and raw starch. With cooked starch, the rate was roughly proportional to the concentration of enzyme over a large range. With raw starch, the maximum rate varied as the square root of the concentration of enzyme. Thus the ratio of maxi-

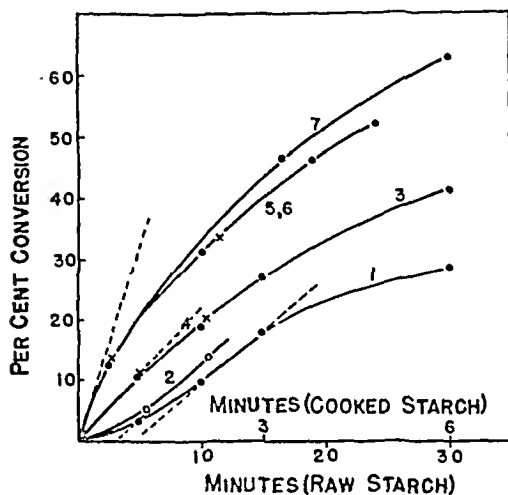


FIG. 3. Initial stages of the hydrolysis of raw starch (Curves 1 to 4) and cooked starch (Curves 5 to 7) at varying maltase concentrations. The concentrations of maltase in Curves 1 to 7 are 0.00, 0.03, 0.32, 1.28, 0.00, 0.32, 1.28 units per ml. respectively;  $\alpha$ -amylase concentration 1.15 units per ml. Saccharifying activity is expressed as per cent conversion of the starch to fermentable sugars.

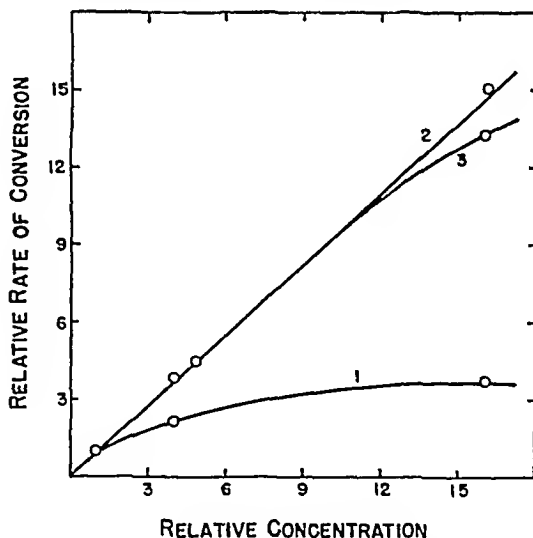


FIG. 4. Relative maximum rates of conversion at varying  $\alpha$ -amylase concentrations for raw starch (Curve 1) and cooked starch (Curves 2 and 3). The maximum rates (set at unity) are 1.9, 2.0, and 32 per cent per minute. The corresponding concentrations of  $\alpha$ -amylase are 1.15, 0.076, and 1.15 units per ml.

num velocities of cooked to raw starch conversion is of one order of magnitude and increases with increasing  $\alpha$ -amylase concentrations.

*Effect of Varying Maltase Concentrations*—The presence of maltase during the conversion of raw starch by  $\alpha$ -amylase resulted in an increase in the over-all rate of conversion and also in the percentage of sugars formed after 5 hours (Table III). For a 2 hour conversion period, shown in Fig. 5, both the rate of conversion and rate of glucose production were constant with respect to maltase concentrations until a concentration of approximately 0.6 unit per ml. was reached. Above this value, the effect of additional maltase became less and less.

The chief effect of maltase at early stages of raw starch conversion was to diminish the apparent lag in sugar production seen with amylase alone. The initial rates of hydrolysis were not, however, much greater than the maxi-

TABLE II

*Digestion of Raw Starch at Varying  $\alpha$ -Amylase Concentrations with and without Added Aspergillus Maltase*

Concentration of enzyme		Per cent conversion after							
		1 hr		2 hrs.		3 hrs		5 hrs.	
$\alpha$ -Amylase	Maltase	Glucose and maltose	Glucose	Glucose and maltose	Glucose	Glucose and maltose	Glucose	Glucose and maltose	Glucose
units per ml.	units per ml.								
0.00	0.32							3.0	2.9
1.15	0.00	36	0	47	0	55	2	64	11
1.15	0.32	54	24	69	42	78	59	90	68
4.60*	0.00							83	16
4.60	0.02	49	10	60	23	72	35	80	41
4.60	0.34	89	33	91	54	84	63	79	71
18.4*	0.00			80	19				
18.4*	0.32	100	53					87	71
18.4	0.06	83	29	98	42	96	41	91	40
18.4	0.38	101	41	100	62	96	67	93	68

\* The enzyme used was salivary  $\alpha$ -amylase (No. III, Table I).

imum rates obtained with  $\alpha$ -amylase alone. In Fig. 3 these rates for Curves 1, 2, and 3 (calculated as the maximum values of the slopes) are 1.7, 1.9, and 2.2 per cent per minute respectively. There was evidently no change in initial rates when the maltase concentration was increased from 0.32 to 1.28 units per ml. The initial rates observed for cooked starch were also practically independent of maltase.

*Effect of Added Sugars and Enzymes*—Maltose inhibited the digestion of raw starch by  $\alpha$ -amylase (Table IV). The addition of 10 mg. per ml. of maltose to raw starch and amylase after 3 hours completely prevented further hydrolysis.

In contrast to maltose, inhibition by glucose occurred during the first half



of the digestion and was not progressive during the latter half. Thus (Table IV) at between 3 and 5 hours the amount of conversion increased 11 per cent in the presence or absence of glucose, whereas in the presence of an

TABLE III  
Conversion of Raw and Cooked Starch at Varying Maltase Concentrations\*

Maltase  units per ml.	Starch	Per cent conversion after							
		1 hr.		2 hrs.		3 hrs.		5 hrs.	
		Glucose and maltose	Glucose	Glucose and maltose	Glucose	Glucose and maltose	Glucose	Glucose and maltose	Glucose
0.00	Raw	36	0	47	0	56	2	64	11
	Cooked	54	0	60	0	64	0	68	3
0.04	Raw	40	9	56	18	64	21	73	24
0.32	Raw	54	24	69	42	78	59	89	68
	Cooked	99	33	100	58	102	67	95	70
0.64	Raw	60	33	82	63	94	71	98	86
	Cooked	102	48	99	63	101	72	95	82

\* The concentration of  $\alpha$ -amylase was constant at 1.15 units per ml.

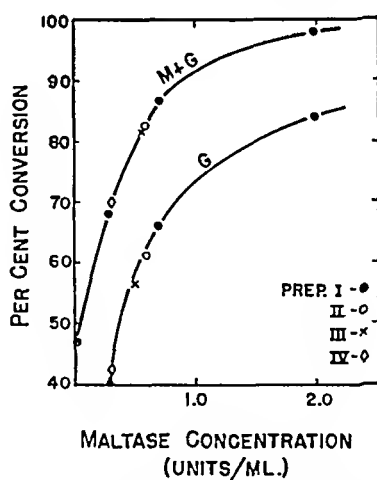


FIG. 5. Conversion of raw starch into glucose ( $G$ ) and into maltose plus glucose ( $M + G$ ) after 2 hours at varying maltase concentrations by four different preparations. The  $\alpha$ -amylase concentration was constant at 1.15 units per ml.

equivalent amount of added maltose an increase of only 7 per cent was observed. The addition of maltase to the digestion mixture after 3 hours resulted in a conversion midway between that obtained with  $\alpha$ -amylase

alone and that with  $\alpha$ -amylase plus maltase present at the start. Glucose did not show an appreciable inhibitory effect in the presence of maltase.

The effect of  $\beta$ -amylase on raw starch is shown in Table IV.  $\beta$ -Amylase I alone digested raw starch to the extent of 3 per cent in 5 hours when enough of it was used to double the rate of hydrolysis of cooked starch by  $\alpha$ -amylase. A mixture of  $\alpha$ - and  $\beta$ -amylases digested raw starch faster than did the  $\alpha$ -enzyme alone, but the addition of  $\beta$ -amylase to the usual mixture of  $\alpha$ -amylase and maltase had no appreciable effect. Wheat extract ( $\beta$ -Amylase II) was in fact inhibitory, as the observations of Kneen and Sandstedt (16) suggest.

When additional  $\alpha$ -amylase was added after 3 hours (Table V), the subsequent hydrolysis was greater than when  $\alpha$ -amylase alone had been added

TABLE IV  
*Effect of Adding and Removing Enzymes and Reaction Products on Course of Conversion of Raw Starch*

Substance added to mixture	Per cent converted after					
	1 hr.	3 hrs.	5 hrs.	1 hr.	3 hrs.	5 hrs.
	$\alpha$ -Amylase			$\alpha$ -Amylase + maltase		
None	36	53	64	54	80	90
Maltose, 10 mg. per ml	30	45	52	45	74	82
Glucose, 10 " " "	28	45	55	52	78	89
$\beta$ -Amylase I (2 mg. per ml.)	40	61	72	44	81	92
Yeast*	42	64	78	59	89	100
" †	28	46	59	28		6
Dialysis*	52	96	100			

\* Percentage of starch rendered soluble (determined by measuring the starch residue remaining).

† Percentage of maltose present.

initially, but not if maltase was also present initially. The effect of added  $\alpha$ -amylase, however, was not as great as the effect of adding more maltase to the system, in which case an acceleration was observed when  $\alpha$ -amylase or  $\alpha$ -amylase with maltase was added initially. An additive acceleration was obtained when additional  $\alpha$ -amylase and maltase were used together in conjunction with the initially added  $\alpha$ -amylase.

*Effect of Yeast and Dialysis*—A sample of fresh bakers' yeast, when incubated under the conditions for digesting raw starch, was found to utilize glucose readily but not maltose (Table VI). Furthermore, the yeast evidently formed glycogen from the glucose so utilized, for reducing sugars appeared only after the yeast residue was boiled with hydrochloric acid (1.2 N, for 2 hours). The substance in the yeast that yielded sugar on acid

hydrolysis could, for the most part, be removed from the yeast by autolysis with toluene for 4 hours.

These observations have been taken into account for the determination of the amount of raw starch digested by  $\alpha$ -amylase in the presence of yeast. 0.1 ml. of the washed yeast suspension was added to the starch digestion mixture at the start of the digestion and at the end of each hour thereafter. At the end of the given times, the reaction was stopped as usual and the

TABLE V

*Effect of Adding Enzymes and Maltose at Later Stages of Raw Starch Digestion*

Added initially	Per cent conversion without further additions		Per cent conversion 2 hrs. after following addition at 3 hrs.				
	3 hrs.	5 hrs.	$\alpha$ -Amylase, 1.15 units per ml.	$\beta$ Amylase, 10.0 mg. per ml.	Maltase, 0.32 unit per ml.	$\alpha$ -Amylase + maltase	Maltose, 10 mg. per ml.
$\alpha$ -Amylase . . . . .	56	64	70	63	80	88	53
" + maltase . . . . .	80	90	90	90	101		
" + maltose . . . . .	30	45			75		
" + glucose . . . . .	28	45			79		

TABLE VI

*Utilization of Maltose and Glucose by Bakers' Yeast*

Each sample contained 0.1 ml. of washed bakers' yeast (10 per cent) suspended in 1 ml. of a solution 0.05 M with respect to acetate buffer and 0.05 per cent with respect to  $\text{CaCl}_2$ .

Treatment of yeast suspension	Per cent sugar			
	Remaining in solution	Appearing in residue	In residue after acid hydrolysis	In residue after autolysis and acid hydrolysis
Incubated with 10 mg. glucose 1 hr. at 45°..	0	0	37	2
" 3 hrs. at 45°; then with glucose 1 hr. . . . .	32			
Incubated with maltose 1 hr. at 45° . . . . .	97			

residue centrifuged and washed. The supernatant liquid and washings were combined and the maltose content thereof determined. No glucose was found in the supernatant liquid. The residue was then autolyzed in 5 ml. of  $\text{H}_2\text{O}$  containing toluene at 40° for 4 hours. The residue was again recovered by centrifugation and hydrolyzed with 1.2 N HCl. It can be seen from Table IV that the presence of yeast increased the over-all rate and extent of digestion both in the absence and in the presence of maltase.

It is rather interesting to note that some maltose still remained in the presence of the maltase after 5 hours.

The hydrolysis of raw starch to completion (at constant  $\alpha$ -amylase concentration) was obtained most rapidly by allowing the reaction to proceed concurrently with dialysis against a solution whose salt concentrations were equal to that of the digestion mixture (Table IV).

#### DISCUSSION

The complementary action of mold bran and pancreas in producing relatively complete and rapid digestion of raw starch can now be attributed to the presence in the mold bran of a non-amylolytic factor. The evidence for the probable identity of this factor with the  $\alpha$ -glucosidase (maltase) of the mold bran is shown in Fig. 5. The "maltase" preparations used possessed varying maltase activities. Three of them were made by procedures so different that it seems unlikely that the non-maltase components would always bear the same relationship to the maltase present; yet in all the preparations, the enhancement of raw starch digestion varies directly with their maltase activity. This evidence, together with the fact that inorganic phosphate was absent during the digestion, makes it unlikely that a phosphorylating mechanism is involved in a manner similar to the synthesis of glycogen by Cori and Cori (17). This synergistic effect of maltase, by no means new, has been discussed under such terminology as "amylase activation" (18) and "complementary action" (19). Meyer and Bernfeld (20) have used yeast maltase in conjunction with  $\beta$ -amylase in the elucidation of the structure of amylopectin.

The behavior of raw starch can be adequately explained, if we postulate four factors contributing to the prevention of complete digestion, namely: permanent inactivation of the enzyme during the course of hydrolysis; inhibition of the  $\alpha$ -amylase by the products of the reaction (particularly by maltose and to a smaller extent by glucose); involvement of maltose in pseudoequilibria with higher polysaccharides; and relative slowness of the hydrolysis of the so called anomalous linkages in the limit dextrins arising from the amylopectin moiety of the starch (21). Although it is usually reported that this 1-6 glucose linkage is not amenable to  $\alpha$ -amylase action, both in the present work and in that of Stark (5) and of Somogyi (21)  $\alpha$ -amylase preparations containing no detectable  $\alpha$ -glucosidase are reported as capable of digesting starch to fermentable sugars. In both these cases the enzyme-substrate ratio may be so much greater than usual that the slow hydrolysis of the 1-6 bonds becomes experimentally appreciable. It is suggested that the small but constant amount of glucose formed may be due to the hydrolysis of "isomaltose" linkages.

When  $\alpha$ -amylase alone is added to the digestion mixture, all four of these

factors contribute to the prevention of complete hydrolysis. Addition of maltose enhances both the substrate inhibition and the resynthesis, whereas with glucose only substrate inhibition is increased. With the addition of maltase, the maltose concentration decreases, thus decreasing both resynthesis and inhibition of the enzyme due to maltose. At the same time the rate of hydrolysis of the 1-6 bonds is accelerated. In the presence of yeast, the inhibiting effect of glucose is also removed, owing apparently to the formation of glycogen. When raw starch is digested with accompanying dialysis, glucose, maltose, and residues carrying 1-6 linkages are removed from the sphere of action so rapidly that even the permanent destruction of enzyme is presumably lessened, resulting in a maximum over-all conversion rate.

The increased action observed in the presence of  $\beta$ -amylase can be attributed to the seission of maltose from the "normal" dextrins present. The fact that  $\beta$ -amylase alone is without action on raw starch is an indication that non-reducing ends of the starch granule are not present at the site of initial enzyme action, inasmuch as the work of Hanes (22), of Myrbäck (23), and of others indicates that the action of  $\beta$ -amylase is from the non-reducing ends of the starch chains. The absence of non-reducing end-groups in the starch granule may be interpreted as due to the presence of strong hydrogen bonding of the hydroxyl group at position 6, as suggested by Caesar (24).

The initial lag in the production of sugar in the presence of  $\alpha$ -amylase alone may be due to the initial exclusive production of dextrins or to this effect and the limited substrate surface available to the enzyme. Somogyi (21) and Myrbäck (23) found for cooked starch that a comparatively extensive hydrolysis to dextrins occurred with  $\alpha$ -amylase before maltose formation began at any considerable rate. The action of maltase in erasing this lag could then be explained by its attacking the 1-6 linkages present in the dextrin.

The lack of linearity exhibited by the maximum rates for raw starch conversion with increasing  $\alpha$ -amylase content may be attributed to the limited substrate surface available to the enzyme at any given time.

There is no evidence in the foregoing experiments that the disintegration of starch granules can be attributed to the removal of a non-starch coating from the surface of the granule. The lack of susceptibility of the surface to enzyme action can better be attributed to the view expressed by Trubell (25) that the starch of the granule is deposited during development by intussusception. Thus the outer layer is deposited first. This results in a highly dehydrated and associated matrix of molecules at the surface of the mature granule. This view has been presented in the work of Wieler (26). Under these circumstances, one is led to the view that the granule will most

probably be attacked at the hilum, where the water content is highest and association by hydrogen bonding is least. The initial stages of the digestion do occur at the hilum. The new surface then exposed to water may be considered as undergoing hydration, and thus becoming more susceptible to enzymic attack.<sup>2</sup>

#### SUMMARY

The complementary action of *Aspergillus oryzae* in the enzymolysis of raw starch by the  $\alpha$ -amylase of pancreas has been traced to the  $\alpha$ -glucosidase (maltase) present in the mold. The effect of varying  $\alpha$ -amylase and maltase concentrations on the extent, course, and initial rate of hydrolysis has been determined. These observations have been compared in turn with the action of the same enzymes on cooked starch. Variations from a standard reaction system were made by increasing the concentration of amylolytic enzymes during the course of hydrolysis, and by adding or removing maltose and glucose.

The observations made have been interpreted to mean that the maltase decreases the operation of the following factors which tend to prevent complete conversion by the  $\alpha$ -amylase: irreversible inactivation; reversible inhibition by maltose; resynthesis from maltose; slow rate of hydrolysis of the "abnormal" linkages present in the amylopectin.

Whereas the difference in the action of  $\alpha$ -amylase on raw and cooked starch seems to be one of rate imposed upon the system by the limited substrate available, the complete lack of susceptibility of the starch granule to attack by  $\beta$ -amylase has been attributed to the masking (by strong hydrogen bonding) of the non-reducing end of the glucose chains.

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<sup>2</sup> The possibility that the amylopectin moiety of the starch matrix may be more easily attacked than the amylose should not be overlooked. Microscopic examination of the disintegration of two varieties of pea starch, one of high amylose content (75 per cent) and one of low amylose content (25 per cent), revealed that the high amylose starch was practically unaffected, whereas the other starch disintegrated quite rapidly under the action of  $\alpha$ -amylase plus maltase. On the other hand, Kerr, Meisel, and Schink (27), who used incomplete raw starch hydrolysis for the preparation of stable corn sirups, conclude that the more easily soluble component of corn-starch is the more easily hydrolyzed.

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# ISOLATION OF STEROIDS FROM THE URINE OF PATIENTS WITH ADRENAL CORTICAL TUMORS AND ADRENAL CORTICAL HYPERPLASIA: A NEW 17-KETOSTEROID, ANDROSTANE-3( $\alpha$ ),11-DIOL-17-ONE

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Information on the metabolism of the steroid hormones has been obtained largely by injection of known amounts of the crystalline hormones and isolation of the steroids which subsequently appeared in the urine (1). Experiments of this type with the adrenal hormones necessarily have been limited in scope by the limited available supply of crystalline adrenal steroids. Some additional information has been obtained by the isolation of steroids from normal or pathologic urine. The urine of patients having hyperactive adrenal cortical lesions is particularly valuable for such studies, since it usually contains abnormally large amounts of steroid material which can be extracted and isolated. The compounds which are finally obtained not only have great theoretic importance but also furnish diagnostic criteria which aid in distinguishing adrenal cortical lesions one from another and from other pathologic lesions elsewhere which produce similar clinical pictures.

Our interest in these particular pathologic conditions was stimulated by the hope that we might be able to isolate the degradation or metabolic products of those important adrenal cortical compounds which carry an oxygen atom at the C-11 position. None of the compounds which have been isolated from either normal or pathologic human urine have this particular chemical feature. We were further motivated by the hope that, if a sufficient number of cases were studied, specific clinical symptoms might be associated with the presence, absence, or quantities of specific urinary steroids.

The isolation studies which have been reported in the literature have shown the presence of the neutral ketones, androsterone, etiocholan-3( $\alpha$ )-ol-17-one, dehydroisoandrosterone, and isoandrosterone in the urine of normal men and women (2-8). The quantities isolated have varied considerably, depending on the techniques used. It appears, however, that the first two substances are present in approximately equal amounts (1.3 to 1.5 mg. per liter) while dehydroisoandrosterone accounts for not more than 10 per cent of the total amount of ketones (4, 5, 8). When an



adrenal cortical tumor is present, there is usually,<sup>1</sup> but not invariably, a greatly increased excretion of androsterone and etiocholan-3( $\alpha$ )-ol-17-one, but the amount of dehydroisoandrosterone excreted is usually greater than the combined amounts of the other ketones (11-15). When cortical hyperplasia is present, the excretion of steroids may be elevated to levels encountered in cases of adrenal tumor but more often the excretion is within normal limits (10-12, 14, 15). In either circumstance, the  $\beta$ -hydroxy-17-ketosteroid fraction (dehydroisoandrosterone and isoandrosterone) is usually, but not invariably (8, 16), much smaller than when an adrenal tumor is present.

In addition to the ketones, the neutral non-ketones, pregnane-3( $\alpha$ ),-20( $\alpha$ )-diol (6, 17) and  $\Delta^5$ -androstene-3( $\beta$ ),16,17-triol (18, 19), have been isolated from normal urine and, in increased amounts, from the urine of patients with adrenal tumors (20-22). The urine of one such patient studied by Hirschmann and Hirschmann (23) also yielded  $\Delta^5$ -androstene-3( $\beta$ ),17( $\alpha$ )-diol and  $\Delta^5$ -pregnene-3( $\beta$ ),20( $\alpha$ )-diol. Pregnane-3( $\alpha$ ),20( $\alpha$ )-diol was isolated by Bütler and Marrian in greater than normal amounts from the urine of women with "adrenal virilism."<sup>2</sup> They also isolated pregnane-3( $\alpha$ ),17,20-triol, a substance which had not been found in normal urine or in the urine of patients with adrenal tumors.

$\Delta^3,5$ -Androstadien-17-one (13, 29) and androsten-17-one (7) have been isolated from urinary extracts, but they are very probably artifacts formed from the alcoholic ketones or their conjugates during the process of hydrolysis (30). Another ketone, an androsten-3( $\alpha$ )-ol-17-one, also probably an artifact, was isolated from the urine of one patient with a cortical tumor (13). 3-Chlorodehydroandrosterone has also been isolated from normal urine (31) and from the urine of patients with adrenal tumors, but it is known to be derived from dehydroisoandrosterone. Other crystalline substances have been isolated in small amounts but not identified.

As the result of the studies on the urine of human subjects which have

<sup>1</sup> A case of adrenal cortical tumor was recently encountered in which the excretion of 17-ketosteroids was only moderately elevated (29 to 30 mg. in 24 hours) and the 3( $\beta$ )-hydroxy-17-ketosteroids (dehydroisoandrosterone and isoandrosterone) constituted only 9 per cent of the total. Three cases have been reported (9, 10) in which normal values for the urinary 17-ketosteroids were associated with cortical tumors.

<sup>2</sup> In their first paper Butler and Marrian (24) stated that the urine which was furnished by L. R. Broster was obtained from women with adrenal tumors. The title of their second paper (25) also stated that the urine was obtained from a woman with an adrenal tumor. Later, (26) they corrected their title by replacing "tumor" with "hyperplasia." The statements in the original paper were not corrected, but a study of the reports of Broster and Vines (27) and of Broster (28) makes it plain that the patients from whom the urine was obtained for the isolation work of Butler and Marrian were suffering from hyperplasia and not from tumors of the adrenal cortex.

been reported, the only substance which, from chemical considerations, conceivably could be related to those adrenal steroids with an oxygen atom at C-11 is the androsten-3( $\alpha$ )-ol-17-one of Wolfe, Fieser, and Friedgood (13). They suggested the possibility that it was the dehydration product of androstane-3( $\alpha$ ),11-diol-17-one. Pregnane-3( $\alpha$ ),20( $\alpha$ )-diol could be derived from progesterone or desoxycorticosterone. Both of these substances have been isolated from adrenal extracts (32-34) and both are known to be sources of urinary pregnanediol (35-39). The adrenal precursor of pregnane-3( $\alpha$ ),17,20-triol is presumably 17-hydroxyprogesterone (40, 41) by analogy with pregnane-3( $\alpha$ ),20( $\alpha$ )-diol and progesterone. The precursor of dehydroisoandrosterone is unknown, but the recent work (42-44) which indicates a relation between cholesterol and the production of adrenal hormones suggests the possibility that the large amounts of dehydroisoandrosterone that are associated with adrenal tumors may be the result of uncontrolled oxidation of cholesterol to this substance by the tumor. The results of a recent study (45) of the metabolism of dehydroisoandrosterone indicate that it is a precursor of androsterone, etiocholan-3( $\alpha$ )-ol-17-one, and  $\Delta^5$ -androsterone-3( $\beta$ ),17( $\alpha$ )-diol. The obvious similarities of structure also suggest dehydroisoandrosterone as the precursor of isoandrosterone and  $\Delta^5$ -androsterone-3( $\beta$ ),16,17-triol. Many of the compounds isolated from the adrenal gland may also be precursors of the 3-hydroxy-17-ketones.

The present report is based on the study of six patients, all women, who had tumors of the adrenal cortex and four additional patients who had bilateral adrenal cortical hyperplasia. The clinical features of these cases eventually will be reported elsewhere. At this time it is sufficient to state that all patients had in varying degrees the endocrine signs and symptoms that accompany such lesions. The quantitative excretion of the urinary 17-ketosteroids in eight of these cases has been reported elsewhere (46). In the present report the same case numbers previously employed are used.

The diagnosis was established at operation in five of the six cases of tumor and in three of the four cases of hyperplasia. The one instance (Case 12) that is classed as a case of tumor but without absolute proof will be discussed later. In three of the cases of hyperplasia the patients were female pseudohermaphrodites. In these cases, the anatomic status and hyperplasia of the adrenal cortex were established by surgical exploration. In the fourth (Case 13), in which exploratory operation was not performed, the patient was a brother of one of the pseudohermaphrodites (Case 9). At the age of 8, he had symptoms of puberty with a 17-ketosteroid excretion of 21 mg. in 24 hours. The urinary steroids which were isolated from his urine were consistent with the conclusion that he was suffering from the same adrenal disease as was his sister.

The methods used in this study were, with some modifications, those described by Callow and Callow (4), and by Wolfe, Fieser, and Friedgood (13). However, instead of working with large volumes of urine during hydrolysis and extraction, 15 to 20 liters of urine were concentrated under reduced pressure to 1.5 to 2 liters, and the concentrated urine was subjected to hydrolysis and extraction. Collection and treatment of the urine are

TABLE I  
*Treatment of Urine*

Case No.	Preservative	Comment
3	HCl*	5 of 15 specimens extracted for pregnanediol; urinary residues and other specimens pooled and stored in cold room with addition of HCl
5	CHCl <sub>3</sub>	Daily specimens pooled and stored in cold room with 10 ml. per liter of CHCl <sub>3</sub>
6	HCl*	Fresh specimens extracted with butyl alcohol; urinary residues acidified and pooled; later, some butyl alcohol extract recombined with urinary residues
7	None	Daily specimens pooled and stored in cold room
8	HCl*	" " extracted with butyl alcohol; extracts reserved for further study; urinary residues acidified and pooled
12	None	Daily specimens pooled and stored in cold room
9	HCl	Urine collected in 5 gallon bottle containing 90 ml. concentrated HCl + 180 ml. water
13	"	Urine collected in 5 gallon bottle containing 90 ml. concentrated HCl + 180 ml. water
10	"	Daily specimens pooled and stored in cold room after addition of HCl
11	"	Urine collected in three 5 gallon bottles each containing 90 ml. concentrated HCl + 180 ml. water; concentrated urine extracted with butyl alcohol and some glucuronides separated from extract; remainder of extract and urinary residue combined for hydrolysis

\* Usually 5 ml. of concentrated HCl were added to a liter of urine; in any event, enough HCl was added to make the urine acid to Congo red.

summarized in Table I. The neutral extract was separated into four major fractions by the method of Wolfe, Fieser, and Friedgood (13). Nothing significant was obtained from the non-alcoholic non-ketonic fraction. Only 3-chlorodehydroandrosterone was obtained from the non-alcoholic ketonic fraction in Cases 5, 6, and 7. The unsaturated artifacts, androsten-17-one and  $\Delta^{3,5}$ -androstadien-17-one, were not encountered. The major substances obtained by fractionation of the alcoholic ketonic and alcoholic non-ketonic fractions on chromatographic

columns of alumina are listed in Table II. A number of other fractions have been isolated but in quantities too small to permit characterization.

When one considers the wide variations in the amounts of urine available and in the concentrations of the steroids and changes in the procedures used, it is evident that, in general, the quantities given in Table II are of doubtful significance for comparison of the steroid excretion in one case with that in

TABLE II

*Summary of Steroid Fractions Isolated from Urine of Patients with Adrenal Cortical Tumors and Hyperplasia*

The results are expressed in mg

Case No	Urine		Neutral extract		Substances isolated												
	Volume	Days			Ketones					Non ketones							
			Total weight	Ketonic fraction	Non ketonic fraction	Dehydroisoandrosterone	Androsterone	Lithocholan 3( $\alpha$ ) ol 17 one	Androstane 3( $\alpha$ ), -11 diol 17 one	Estrone	Pregnane 3( $\alpha$ ), 20 ( $\alpha$ ) diol	Pregnane-3( $\alpha$ ), 17, -20 triol	$\Delta^1$ Androstene 3-( $\beta$ ), 17( $\alpha$ ) diol	$\Delta^1$ Androstene 1-( $\beta$ ), 16, 17-triol	Pregnane 3( $\beta$ ), 20-( $\alpha$ ) diol	C <sub>27</sub> H <sub>48</sub> O <sub>2</sub> (II)	C <sub>27</sub> H <sub>48</sub> O <sub>2</sub> (I')
Patients with adrenal tumors																	
3			14	1450	570	6400	184		65	30			28		20		
5	43.0	26	9300	5600	2900	2190	49	192	11	62	107		55	10			20
6	6.4	13	2920	1670	710	894		33			53						
7	5.7	3	1570	870		391		13			13						
8	50.4	31	8000	3500	3000	740	290	127	77		90		10				22
12	15.0	13	3200	1250	1630		55				127	47					
Patients with adrenal hyperplasia																	
9	18.0		1200	320	590							27					88
13	18.0		1160	520	470	33	55		65		Trace	35			12		105
10	11.0	13	1350	410	410		19	11	23		71						
11	54.0		4480	2010	1650	7	122		80		219	74			6		131

another. These quantities, however, may be used for comparison with other quantities in the same case with reservations as to unknown losses during the isolation procedures. The data in Case 6 are not complete, owing to losses during an attempt to isolate the conjugated steroids. The only conjugate isolated was sodium dehydroisoandrosterone sulfate; the amount is included in Table II as dehydroisoandrosterone. The substances isolated from the urine in Case 8 are those that were not extracted from the unhydrolyzed urine by butyl alcohol. The substances isolated from the

butyl alcohol extract will be described in a later report. The ketonic fractions in Cases 9 and 13 were accidentally combined.

Dehydroisoandrosterone was isolated in large amounts from the urine of all except one of the patients with adrenal tumors, but in small amounts or not at all from the urine of the patients with hyperplasia. The urine in Case 8 was unique in that 17 gm. of dehydroisoandrosterone were isolated from the butyl alcohol extract as sodium dehydroisoandrosterone sulfate; this quantity is not included in Table II. These results are in agreement with those of other investigators and support the suggestion of Crooke and Callow that the excretion of large amounts of dehydroisoandrosterone is indicative of the presence of an adrenal cortical tumor.

Androsterone and etiocholan-3( $\alpha$ )-ol-17-one were not found consistently, although the latter substance was isolated from the urine in all but one of the cases of tumor. Androsterone was found in the urine of all of the patients with hyperplasia, whereas etiocholan-3( $\alpha$ )-ol-17-one was found in the urine of only one of these patients. These results are in general agreement with the quantitative results of Dobriner and his associates (8). Their one patient with an adrenal tumor excreted very little androsterone, while two of their patients with hyperplasia excreted very little etiocholan-3( $\alpha$ )-ol-17-one.

The ketone which is formulated as androstane-3( $\alpha$ ),11-diol-17-one or 11-hydroxyandrosterone (I) (Fig. 1) is the only substance with an oxygen atom at C-11 that has been isolated from human urine. A preliminary report of its identification as an 11-oxygenated steroid has been made (47). Marker and his associates (48-50) have described two alcoholic  $C_{21}$  compounds which were presumed but not proved to contain an oxygen atom at C-11 and which were obtained from the urine of pregnant mares. Dobriner and associates (8) described a ketone with a melting point of 199-200° to which they assigned the formula,  $C_{19}H_{32}O_3$ . It was obtained from two collections of urine from normal persons and one collection from a patient with evidence of adrenal hyperplasia. It may be that this substance is androstane-3( $\alpha$ ),11-diol-17-one.

Analysis of our ketone and its acetate indicated the formula  $C_{19}H_{30}O_3$  which would permit the presence of two hydroxyl groups in addition to the known ketone group. Since it formed a monoacetate,  $C_{21}H_{32}O_4$ , when heated at 90° with pyridine and acetic anhydride, it seemed probable that the 3rd atom of oxygen was present as a hydroxyl group in a highly hindered position, although the possibility that it was present as a ketone group was not excluded. The positions of the 3 oxygen atoms were definitely established by oxidation of the alcoholic ketone (I) to androstane-3,11,17-trione (II) which has been prepared several times from adrenal steroids (51, 52). The identity of the oxidation product was established by

comparison with a specimen of androstane-3,11,17-trione, which had been prepared from an adrenal steroid, and by comparison of their oximes.

The color produced by the urinary ketone (I) in the Zimmermann reaction was characteristic of the 17-ketones. There was maximal absorption at 520  $m\mu$  and the absorption curve was practically identical with that obtained with dehydroisoandrosterone. The intensity of the color, however, was only 74 per cent of the intensity developed by an equi-

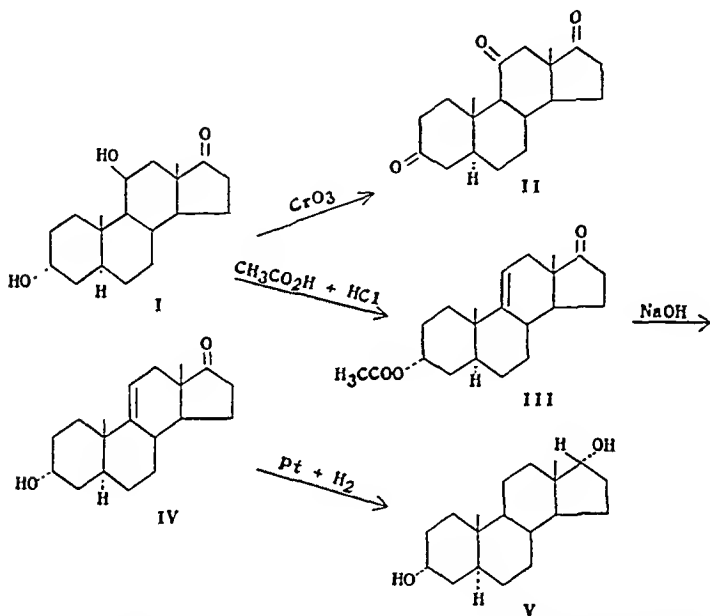


FIG. 1. Conversion of androstane-3(α),11-diol-17-one to androstane-3,11,17-trione and androstane-3(α),17(α)-diol. The solid line which connects the hydroxyl group to C-11 is not intended to imply a  $\beta$  configuration.

molecular amount of dehydroisoandrosterone. Androstane-3,11,17-trione (II) produced 117 per cent as much color as dehydroisoandrosterone.

With the ketone group thus placed at C-17, the hydroxyl group which could be acetylated must be at C-3. It remained to establish the function of the oxygen atom at C-11. Since a hydroxyl group was indicated by the analyses, the urinary substance was refluxed for 15 minutes in a mixture of acetic and hydrochloric acids (4:1). Shoppee (53) and Shoppee and Reichstein (54, 55) have shown that this treatment removes an 11-hydroxyl group with the formation of a double bond. The main product of this

reaction was an androsten-3( $\alpha$ )-ol-17-one acetate (III) which melted at 190–192°;  $[\alpha]_D^{25} = +135^\circ \pm 3^\circ$ . Hydrolysis in alkaline solution gave the androsten-3( $\alpha$ )-ol-17-one (IV) which melted at 189–190°;  $[\alpha]_D^{26} = +140^\circ \pm 2^\circ$ . It was reduced with platinum and hydrogen in acetic acid to androstane-3( $\alpha$ ),17( $\alpha$ )-diol (V) which melted at 219–220°; the diacetate melted at 158–160°. The identity of the product of reduction and its acetate was established by comparison with androstane-3( $\alpha$ ),17( $\alpha$ )-diol and its diacetate, which were prepared from androsterone by the method of Butenandt and Tscherning (56).

This series of reactions definitely established the presence of a hydroxyl group at C-11. At the same time, the  $\alpha$  configuration of the C-3 hydroxyl group was established. Accordingly, the urinary substance was formulated as androstane-3( $\alpha$ ),11-diol-17-one (I). Assignment of a configuration to the 11-hydroxyl group is not possible with the information at hand.

It was anticipated that loss of the 11-hydroxyl group might yield the same androsten-3( $\alpha$ )-ol-17-one that was obtained from the urine of a woman with an adrenal tumor by Wolfe, Fieser, and Friedgood (13). However, their androstenolone melted at 181–183° and  $[\alpha]_D^{20.5} = +122^\circ \pm 2^\circ$ . The acetate of their compound melted at 178–180° and  $[\alpha]_D^{23.6} = +114^\circ \pm 5^\circ$ . The discrepancies in the melting points and specific rotations indicate that the androstenolones are not identical, but further investigation will be necessary to determine whether or not the differences are significant. It is possible that the androstenolone of Wolfe, Fieser, and Friedgood is isomeric with ours with respect to the double bond, or that it is a mixture of the isomers.

The position of the double bond in our androstenolone is uncertain but, in accord with the conclusions of Shoppee and Reichstein (55), it is placed tentatively at 9:11 rather than at 11:12. There is also the possibility that the product was a mixture of androstenolones isomeric with respect to the double bond. Chromatographic analysis, however, gave no indication of the presence of more than one androstenolone.

Androstane-3( $\alpha$ ),11-diol-17-one conceivably could be derived from any of the adrenal steroids with an oxygen atom at C-11, but it is more likely that it would be derived from those adrenal steroids which also have a hydroxyl group at C-17 such as 17-hydroxycorticosterone. Von Euw and Reichstein (41) isolated the C-3 epimer, androstane-3( $\beta$ ),11-diol-17-one, from adrenal extracts. This substance and adrenosterone ( $\Delta^4$ -androsterone-3,11,17-trione) are also possible precursors.

The non-ketonic fraction of the urinary steroids has been largely neglected. Although Butler and Marrian (24) isolated pregnanediol, pregnanetriol, and an unidentified triol from the urine of women with adrenal cortical hyperplasia, it is only recently that Hirschmann and Hirschmann

(23) have described a chromatographic study of this fraction derived from the urine of a boy with an adrenal tumor. In the present study, pregnane-3( $\alpha$ ),20( $\alpha$ )-diol was found in the urine in all but one of the cases. It is considered to be derived from adrenal products, since ovarian function appeared to be completely suppressed in all of the women. An effort was made to isolate sodium pregnanediol glucuronide from the urine of five patients (Cases 3, 5, 6, 8, and 11) by Venning's method (57). In only two cases (Nos. 3 and 11) was it possible to do so. In one instance (Case 11) the sodium pregnanediol glucuronide was accompanied by sodium pregnane-3( $\alpha$ ),17,20-triol glucuronide. The mixture, which also contained approximately 25 per cent of the glucuronides of hydroxyketo-steroids, melted at 250–252°. The melting point was not changed by repeated crystallizations. The glucuronides were subjected to enzymic hydrolysis and the mixture of steroids was separated into ketonic and non-ketonic fractions. The ketonic fraction was further separated into several small fractions which could not be identified. The non-ketonic fraction was separated into pregnane-3( $\alpha$ ),20( $\alpha$ )-diol and pregnane-3( $\alpha$ ),-17,20-triol in the approximate ratio of 2:3.

Pregnanediol has been found in the urine of women with adrenal tumors and hyperplasia by Butler and Marrian (24), Venning, Weil, and Browne (58), Salmon, Geist, and Salmon (20), Genitis and Bronstein (59), and Anderson, Hain, and Patterson (21). The quantities of pregnanediol which were isolated suggest that these diseases of the adrenal cortex result in a relatively large production of progesterone or of desoxycorticosterone or both.

The presence of pregnane-3( $\alpha$ ),17,20-triol in the urine of the four patients with hyperplasia and its absence from the urine of all but one patient with a tumor suggest that the excretion of this substance may be characteristic of hyperplasia. Butler and Marrian (24) first isolated this substance from the urine of two women with adrenal cortical hyperplasia. They, as well as Broster and Vines (27), defended the thesis that it is characteristic of "adrenal virilism" since it had not been found in normal urine. However, the term "adrenal virilism" scarcely can be said to have a specific meaning and can be construed to refer to symptoms which may be caused by a cortical tumor or by cortical hyperplasia. Up to the present time no evidence has been forthcoming as to the possible occurrence of this pregnanetriol in the urine of patients with cortical tumors. If the presence of any compound in the urine could be established as characteristic of hyperplasia or of a tumor exclusively, a valuable tool for differential diagnosis would be available. When one considers the protean symptoms of adrenal cortical tumors, it seems very unlikely that such a compound will be discovered. Although pregnanetriol was found in the urine in



Case 12, in which the lesion was classified as a cortical tumor, the presence of the cortical tumor was not verified by exploration or at necropsy. The patient, however, had a mass overlying the right kidney and there were masses in the lungs that had the roentgenologic characteristics of metastatic malignant lesions. She had most of the clinical features of Cushing's syndrome, and, in addition, many of those that have been described under the term "adrenal virilism." In this case there is the possibility that the patient had adrenal cortical hyperplasia and an incidental non-adrenal malignant lesion. Clinically, this possibility seems far fetched. The fact that dehydroisoandrosterone was not found in the urine and that pregnane-3( $\alpha$ ),17,20-triol was found, assuming that she did have an adrenal cortical tumor, becomes exceedingly important and indicates that chemical criteria for the differential diagnosis of adrenal cortical tumor and hyperplasia are not absolute. The cases previously mentioned in which the urinary excretion of 17-ketosteroids was not increased constitute further evidence for this view. Although only a limited number of cases have been studied, it appears likely that most patients having functioning adrenal cortical tumors excrete dehydroisoandrosterone in the urine, that most patients having adrenal cortical hyperplasia excrete pregnane-3( $\alpha$ ),17,20-triol, but that exceptions to this general rule do occur. In view of the quantitative and qualitative differences in the symptoms caused by these hyperfunctioning lesions, such exceptions are not surprising.

Two isomeric non-ketones,  $C_{19}H_{32}O_2$ , with similar melting points were isolated. The one designated as  $C_{19}H_{32}O_2$  (H.) was obtained only from the patients with hyperplasia, the other,  $C_{19}H_{32}O_2$  (T.), only from patients with tumors. Their respective melting points were 203–205° and 207–209°. The melting point of a mixture was depressed to about 180°. The acetate and benzoate of  $C_{19}H_{32}O_2$  (H.) were prepared but they could not be crystallized. Oxidation of  $C_{19}H_{32}O_2$  (T.) with chromic acid gave a small amount of crystalline product which melted at 112–113°. Sufficient material was not available for further study.

$\Delta^5$ -Androstene-3( $\beta$ ),17( $\alpha$ )-diol was isolated from the urine in three cases of adrenal tumor. It was not found in the urine in the cases of hyperplasia in which dehydroisoandrosterone was present in very small amounts or not at all. This result is in agreement with the suggestion (23, 45) that  $\Delta^5$ -androstene-3( $\beta$ ),17( $\alpha$ )-diol is a metabolic product of dehydroisoandrosterone, although other possibilities cannot be excluded.

Another non-ketone which has been obtained in small amounts from the urine of two patients with adrenal hyperplasia has been tentatively identified as pregnane-3( $\beta$ ),20( $\alpha$ )-diol, since its melting point of 180–182° and the melting point of its acetate, 142–143°, agree with the recorded (60) melting point (182°) of pregnane-3( $\beta$ ),20( $\alpha$ )-diol and its diacetate (141°).

The presence of a 3( $\beta$ )-hydroxyl group was indicated by the formation of a precipitate with digitonin in 90 per cent methanol.

Since Hirschmann (22) described the isolation of  $\Delta^5$ -androstene-3( $\beta$ ),16,-17-triol in 1943, a search for this compound has been made. It is so readily isolated that it seems unlikely that it could have been present in appreciable amounts in the urine examined. It was found eventually in the alcoholic non-ketonic fraction in Case 5, which was eluted with a mixture of acetic acid, pyridine, and dry ether (2:20:78). The total weight of crude crystalline material was 10.3 mg. It was accompanied by a large amount of gum.  $\Delta^5$ -Androstene-3( $\beta$ ),16,17-triol has since been found in relatively large amounts in the urine of two women with adrenal tumors and extensive metastatic tumors in the liver. The steroids of these two patients will be described in a later report.

Several cases have been reported (61-63) in which adrenal tumors were associated with greatly increased urinary excretions of estrogens as determined by bioassay. The urine in one case, in which the patient was a man who had become feminized, was investigated by Burrows, Cook, Roe, and Warren (29), who were unable to isolate any crystalline estrogen. The only case that we have encountered in which there was a high urinary estrogenic titer was Case 5. The extract of the pooled urine (26 days) contained 88,800 rat units of estrogen which corresponds to an average excretion of 3400 rat units per day. The phenolic fraction of the extract was separated into fractions containing the "weak" phenols and the "strong" phenols. The "weak" phenols were further separated into those with ketone groups and those without such groups, by means of Girard's reagent. The ketonic "weak" phenol fraction yielded 62 mg. of estrone. The non-ketonic "weak" phenol fraction gave a few crystals which melted at about 130°. Estradiol could not be identified in this fraction. Estriol could not be isolated from the "strong" phenol fraction.

#### EXPERIMENTAL

The treatment of the urine during collection has been outlined in Table I. The pooled specimens were concentrated to approximately a tenth of their volume. In four instances (Cases 3, 5, 7, and 12), the concentrated urine was extracted with ether or benzene before hydrolysis. The material thus obtained was, respectively, 8, 7, 13, and 33 per cent of the total neutral extract. Dehydroisoandrosterone was isolated from this preliminary extract of the urine in Cases 5 and 7. In Cases 3 and 12 this extract was combined with the main extract for fractionation.

An aliquot of the concentrated urine was titrated with  $N$  NaOH and Congo red indicator. The urine was then heated to boiling and enough concentrated HCl was added to bring to normal the concentration of acid which

could be titrated to Congo red. Boiling was continued for 10 minutes. The urine was then cooled under the tap and thoroughly extracted with benzene or ether or both. In the later work, the boiled urine was extracted three times with 500 ml. of benzene, three times with 500 ml. of ether, and twice with 500 ml. of chloroform. The extracts were washed once separately with 10 per cent NaOH. The benzene and chloroform were distilled almost to dryness, and the residues were combined with the ether extract which had been concentrated to 400 to 500 ml. This ether solution was washed twice with 50 ml. of 10 per cent NaOH, twice with 50 ml. of NaOH containing 1 gm. of sodium hydrosulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ), and then with water until neutral. The ether was dried and distilled and the residue weighed.

*Fractionation of Extract*—The total neutral extract was separated into ketonic and non-ketonic fractions with the aid of Girard's Reagent T. The non-ketonic fraction was assayed for 17-ketosteroids. If appreciable amounts were found, the treatment with Girard's reagent was repeated. Each fraction was separated into alcoholic and non-alcoholic subfractions with the aid of succinic anhydride and pyridine (13). This procedure was found to be very satisfactory if sufficient anhydride was used. Twice the weight of the fraction or approximately 6 molecular equivalents were sufficient.

*Neutral Ketonic Fraction*—In three instances (Cases 5, 6, and 7), the non-alcoholic ketonic fraction yielded 3-chlorodehydroandrosterone by crystallization from methanol. This substance is an artifact and is included in the amount of dehydroisoandrosterone reported as isolated. The non-alcoholic fraction was subjected to chromatographic analysis in Cases 6, 7, and 12. Nothing crystalline was obtained other than chlorodehydroandrosterone.

The procedure for chromatographic analysis of the alcoholic ketonic fraction was that used by Callow and Callow (4). The alumina was the product manufactured by E. Merek (Darmstadt), standardized according to Brockmann. The concentration of alcohol in carbon tetrachloride was increased progressively from 0.1 to 10 per cent by volume. The substances are listed in Table III, according to the order in which they were eluted from the columns. Many of the fractions so obtained contained only material which could not be made to crystallize. They were discarded after exhausting every means to obtain crystals or a crystalline derivative. Most of the fractions that appeared to be entirely crystalline contained some amorphous material which was removed by recrystallization. The fractions which were partially crystalline and obviously contained much amorphous material were dissolved in a relatively large volume of acetone, and an equal volume of water was added. Partial or complete evaporation of the acetone resulted in the formation of crystals, while the amorphous

material remained largely in suspension. The crystals could be separated readily by slow centrifugation or by filtration. This procedure afforded a convenient preliminary purification.

*Melting Points*—The melting points below 250° were determined with the Fisher-Johns apparatus. They are recorded as read. The melting points above 250°, except where indicated, were determined with a gas-heated block and are corrected for stem exposure.

*3-Chlorodehydroandrosterone*—The extract of the urine in Case 5 gave the largest amount of 3-chlorodehydroandrosterone. From 1.86 gm. of the non-alcoholic ketone fraction 640 mg. of crystals were obtained by recrystal-

TABLE III

*Urinary Steroids and Solvents Used for Their Elution from Chromatographic Columns*

Substance	Solvent used for elution
Ketones	
Dehydroisoandrosterone...	CCl <sub>4</sub> and CCl <sub>4</sub> + 0.1% alcohol
Androsterone.....	" + 0.1% alcohol
Etiocolan-3(α)-ol-17-one.....	" + 0.1-0.2% alcohol
Androstane-3(α),11-diol-17-one.....	" + 0.4-0.5% "
Non-ketones	
Pregnane-3(β),20(α)-diol. ...	Benzene + 0.1-0.2% alcohol
Δ <sup>4</sup> -Androstene-3(β),17(α)-diol....	" + 0.2-0.4% "
Pregnane-3(α),20(α)-diol.....	" + 0.2-0.5% "
C <sub>19</sub> H <sub>32</sub> O <sub>2</sub> (H.) ...	" + 0.3-0.5% "
" (T.) ...	" + 0.3-0.5% "
Pregnane-3(α),17,20-triol..	" + 4% alcohol
Δ <sup>4</sup> -Androstene-3(β),16,17-triol	Acetic acid, pyridine, dry ether, 2, 20, and 78 parts by volume

lization from methanol. They melted at 156-157° and  $[\alpha]_D^{25} = +19^\circ \pm 1.1^\circ$  ( $c = 0.890$  in alcohol). A qualitative test showed the presence of chlorine. Butenandt and Dannenbaum (31) gave a melting point of 157° and  $[\alpha]_D^{20} = +15.5^\circ$  for 3-chlorodehydroandrosterone. For identification, 44 mg. were heated at 150° for 3 hours with 300 mg. of sodium benzoate and 2 gm. of benzoic acid. The cooled mixture was treated with water and NaOH and filtered. The insoluble residuc was crystallized from methanol. It melted at 258° (corrected) and the melting point was not depressed by admixture of dehydroisoandrosterone benzoate.

*Dehydroisoandrosterone, Androsterone, and Etiocolan-3(α)-ol-17-one*—These substances were eluted from the column in the order given. Their purification and properties have been described sufficiently by Callow and

Callow (4, 5) and others (6, 7, 13). Their identities were established by comparison of the purified fractions and their acetates or benzoates with the corresponding authentic specimens. Addition of one substance to either of the other two resulted in a marked depression of the melting point.

*Androstane-3( $\alpha$ ),11-diol-17-one (11-Hydroxyandrosterone)*—This substance was eluted from the column of alumina by carbon tetrachloride containing 0.4 to 0.5 per cent of alcohol. It was always well separated from the etiocholan-3( $\alpha$ )-ol-17-one fraction. It was readily purified by recrystallization from acetone and melted at 197–198°.

$C_{19}H_{30}O_3$ . Calculated, C 74.44, H 9.87; found, C 74.24, H 10.19  
 $[\alpha]_D^{25} = +96.6^\circ \pm 2^\circ$  ( $c = 0.590$  in alcohol)

*Androstane-3( $\alpha$ ),11-diol-17-one Acetate*—The acetate was prepared from each specimen isolated for purposes of comparison. For analysis, 25 mg. were heated at 90° for 30 minutes with 0.5 ml. of pyridine and 0.2 ml. of acetic anhydride. The solution was cooled in ice water while an excess of cold dilute HCl was added. The monoacetate, which separated in crystalline form, was filtered out, washed with dilute HCl and water, dried, and recrystallized from acetone. It crystallized as long rods (18 mg.) which melted at 238–240°.

$C_{21}H_{32}O_4$ . Calculated, C 72.38, H 9.26; found, C 72.77, H 9.17

*Oxidation of Androstane-3( $\alpha$ ),11-diol-17-one to Androstane-3,11,17-trione*—To a solution of 31 mg. (0.1 mm) of substance in 3 ml. of glacial acetic acid was added 0.466 milliequivalent of chromic acid in 0.5 ml. of 90 per cent acetic acid. After the solution had stood overnight, the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate and washed free of acid with a solution of sodium bicarbonate. Evaporation of the solvent left 30 mg. of neutral material which was recrystallized from dry ether. Two crops of crystals weighed 23 mg. and melted at 178–179°.

$C_{19}H_{26}O_3$ . Calculated, C 75.46, H 8.67; found, C 75.98, H 9.00  
 $[\alpha]_D^{25} = +151^\circ \pm 3^\circ$  ( $c = 0.383$  in acetone)

These properties coincided with those of androstane-3,11,17-trione which had been prepared from adrenal steroids (51) and which melted at 178–179°;  $[\alpha]_D^{25} = +152^\circ \pm 2^\circ$  ( $c = 0.570$  in acetone). A mixture of the two preparations melted at 178–179°. Von Euw and Reichstein (64) give a slightly higher melting point (182–183°) for this triketone and  $[\alpha]_D^{12} = +152.8^\circ \pm 5^\circ$  ( $c = 0.422$  in acetone).

The oximes of these two preparations were prepared in the usual way.

After recrystallization from methanol both specimens of oxime and a mixture of them melted at 264–268° (decomposition).

*Dehydration of Androstane-3( $\alpha$ ),11-diol-17-one*—A solution of 30 mg. in 2 ml. of glacial acetic acid and 0.5 ml. of concentrated HCl was refluxed 15 minutes. The solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate and washed free of acid with a solution of sodium carbonate. The solvent was evaporated and the residue was chromatographed in 10 ml. of  $\text{CCl}_4$  on a column of 1.5 gm. of alumina (8 by 70 mm.). The first seven 10 ml. portions of  $\text{CCl}_4$  eluted 26 mg. of crystals which melted at 191–192° after crystallization from acetone;  $[\alpha]_D^{25} = 135^\circ \pm 3^\circ$  ( $c = 0.340$  in 95 per cent alcohol). This large fraction proved to be androsten-3( $\alpha$ )-ol-17-one acetate.

$\text{C}_{21}\text{H}_{32}\text{O}_2$ . Calculated, C 76.32, H 9.15; found, C 76.27, H 9.09

3 mg. of androsten-3( $\alpha$ )-ol-17-one were eluted from the column by carbon tetrachloride containing 0.5 per cent of alcohol. After recrystallization of this substance from dilute methanol, its melting point of 184–186° was not depressed by admixture of the free alcohol described in the next paragraph.

*Androsten-3( $\alpha$ )-ol-17-one*—For hydrolysis, 26 mg. of the acetate were dissolved in 15 ml. of methanol and 3 ml. of  $\text{N NaOH}$ . After standing overnight the solution was diluted with water and extracted with carbon tetrachloride. The extract was washed with water, dried with  $\text{Na}_2\text{SO}_4$ , and evaporated to dryness. The residue was recrystallized from ethyl acetate. The melting point was then 187–189°;  $[\alpha]_D^{25} = +140^\circ \pm 2^\circ$  ( $c = 0.500$  in 95 per cent alcohol). A mixture with the acetate melted at 150–155°. Reacetylation of a small portion with acetic anhydride and pyridine gave the acetate with a melting point of 188–190°. When mixed with the substance formed by the dehydration procedure, it melted at 188–191°.

*Hydrogenation of Androsten-3( $\alpha$ )-ol-17-one to Androstane-3( $\alpha$ ),17( $\alpha$ )-diol*—The catalyst, 25 mg. of platinum oxide, and 5 ml. of glacial acetic acid were shaken with hydrogen until uptake ceased. Then 12.4 mg. of the androstenolone were added and shaking was continued until there was no further uptake of hydrogen. The hydrogen absorbed was 1.84 ml.; calculated for 2 moles, 1.92 ml. Another 25 mg. of fresh catalyst were added and shaking was continued for 4 hours. The catalyst was removed by filtration and the acetic acid by distillation under reduced pressure. The residue, after recrystallization from acetone, melted at 219–220° and a mixture with androstane-3( $\alpha$ ),17( $\alpha$ )-diol (m.p. 219–220°) melted at 219–220°.

The acetate was prepared with pyridine and acetic anhydride. It was

recrystallized from dilute methanol and melted at 157–158°. A mixture with androstane-3( $\alpha$ ),17( $\alpha$ )-diol diacetate (56) (m.p. 159–160°) melted at 157–160°.

*Androstane-3( $\alpha$ ),11-diol-17-one and Androstane-3,11,17-trione in Zimmermann Reaction*—For the Zimmermann reaction, 0.2 ml. portions of absolute alcoholic solutions of androstane-3( $\alpha$ ),11-diol-17-one, androstane-3,11,17-trione, and dehydroisoandrosterone which contained respectively 0.902, 0.901, and 1.041 micromoles per ml., were treated with equal volumes of 2 per cent *m*-dinitrobenzene and 2.5 *M* KOH in absolute alcohol. After development of the color for 45 minutes at 25°, the respective colorimetric readings were 175, 277, and 274. With the dehydroisoandrosterone as a standard, the first two readings corresponded to 0.665 and 1.052 micromoles per ml. respectively. When these values were divided by the respective concentrations, it was found that, molecule for molecule, androstane-3( $\alpha$ ),11-diol-17-one produced 74 per cent and androstane-3,11,17-trione 117 per cent as much color as dehydroisoandrosterone.

The colored solutions were also analyzed in a Beckman spectrophotometer. Maximal absorption was found at 520  $m\mu$  for all three substances.

*Neutral Non-Ketonic Fraction*—The chromatographic analysis of this fraction was similar to that of the ketonic fraction, except that benzene was used in place of carbon tetrachloride and the fraction was put on the column in a mixture of benzene and petroleum ether. A final elution was made in two instances (Cases 5 and 11) with a mixture of acetic acid, pyridine, and dry ether (2:20:78 parts by volume).

*Isolation of  $\Delta^5$ -Androstene-3( $\beta$ ),17( $\alpha$ )-diol*—The largest amount of this substance was obtained from the urine in Case 5. It was eluted with benzene containing 0.4 per cent alcohol. This fraction (120 mg.) was rechromatographed on a column of alumina with carbon tetrachloride-alcohol mixtures. The fractions that were eluted with 0.3 to 1 per cent alcohol in carbon tetrachloride were combined (55 mg.) and recrystallized from methanol and acetone to a constant melting point of 178–180°;  $[\alpha]_D^{27} = -53.2^\circ \pm 2.8^\circ$  ( $c = 0.357$  in alcohol). The crystals (38 mg.) gave a crystalline precipitate with digitonin in 90 per cent methanol. A mixture with  $\Delta^5$ -androstene-3( $\beta$ ),17( $\alpha$ )-diol (m.p. 177–178°;  $[\alpha]_D^{27} = -51.4^\circ \pm 2.5^\circ$ ) prepared from dehydroisoandrosterone (65) melted at 177–178°.

*$\Delta^5$ -Androstene-3( $\beta$ ),17( $\alpha$ )-diol Diacetate*—5 mg. of  $\Delta^5$ -androstene-3( $\beta$ ),17( $\alpha$ )-diol, 5 drops of pyridine, and 3 drops of acetic anhydride were heated at 90° for 30 minutes. After the mixture had cooled, dilute HCl was added. The precipitate was filtered out, washed with water, and crystallized from methanol. The acetate crystallized as plates which

melted at 159–160°. Admixture with authentic  $\Delta^5$ -androstene-3( $\beta$ ),17( $\alpha$ )-diol diacetate (m.p. 159–160°) did not depress the melting point.

$C_{21}H_{34}O_4$ . Calculated, C 73.78, H 9.15; found, C 73.81, H 9.47

Corresponding fractions in Cases 3 and 8 and their acetates were also compared with the authentic specimens and thus identified.

*Isolation of  $\Delta^5$ -Androstene-3( $\beta$ ),16,17-triol*—Since considerable of the weight of the non-ketonic fraction in Case 5 remained unaccounted for after the column of alumina had been washed with 10 per cent alcohol in benzene, elution was continued with a mixture of acetic acid, pyridine, and dry ether (2:20:78). The greatest weight (170 mg.) was obtained with the third 100 ml. portion of this solvent. When this fraction was allowed to stand for some time in a small volume of methanol, 10.3 mg. of crystals separated. Recrystallization from methanol gave 2.7 mg. of crystals, which melted at 264–266° (corrected). Hirschmann (22) gave a melting point of 265–270°. The crystals were acetylated at 90° with a few drops of pyridine and acetic anhydride. The product, crystallized from dilute methanol, melted at 184–185°. A mixture with a specimen of  $\Delta^5$ -androstene-3( $\beta$ ),16,17-triol triacetate (m.p. 186–187°), kindly supplied by Dr. H. Hirschmann, melted at 184–186°.

*Isolation of Pregnane-3( $\alpha$ ),20( $\alpha$ )-diol*—In some cases this substance was not eluted from the column as a clear cut fraction. It was associated with the two compounds  $C_{19}H_{32}O_2$  when they were present, but could be separated from them by a second chromatographic analysis. In this second analysis, pregnanediol was eluted from the column before  $C_{19}H_{32}O_2$  (H.) and after  $C_{19}H_{32}O_2$  (T.). The pregnanediol was recrystallized from methanol. It melted at 237–239°. Not all of the preparations were highly purified. As soon as it became evident that the fraction probably was pregnanediol, a portion was converted to the acetate which was purified for comparison with an authentic specimen of pregnanediol diacetate. This procedure was desirable because mixtures of pregnanediol and pregnane-3( $\alpha$ ),17,20-triol have the melting point of pregnanediol. The acetates of the two substances, however, show the usual depression of their melting points when mixed. The first preparations of pregnanediol and its diacetate were purified, compared with authentic specimens, analyzed, and used as reference compounds for subsequent preparations.

Pregnane-3( $\alpha$ ),20( $\alpha$ )-diol.  $C_{21}H_{36}O_2$ . Calculated. C 78.70, H 11.33  
Found. " 78.97, " 11.65

Pregnane-3( $\alpha$ ),20( $\alpha$ )-diol diacetate.  $C_{23}H_{40}O_4$ . Calculated. C 74.22, H 9.97  
Found. " 74.32, " 10.0



*Isolation of Pregnane-3( $\alpha$ ),17,20-triol*—The fractions that were removed from the columns with benzene containing 4 per cent of alcohol, after several recrystallizations from methanol, melted at 250–252° (Fisher-Johns apparatus);  $[\alpha]_D^{28} = -3.9^\circ \pm 1.9^\circ$  ( $c = 0.517$  in 95 per cent alcohol).

$C_{21}H_{32}O_3$ . Calculated, C 74.93, H 10.79; found, C 75.07, H 10.99

The diacetate was prepared in the cold with acetic anhydride and pyridine. After repeated crystallization from methanol it melted at 156–158°.

$C_{23}H_{40}O_5$ . Calculated, C 71.38, H 9.59; found, C 71.34, H 9.69

Butler and Marrian (24) originally gave 243–244° and 136.5° as the melting points of pregnane-3( $\alpha$ ),17,20-triol and its diacetate. Later (25) they revised the melting point of the acetate to 150–151°.

*Isolation of  $C_{19}H_{32}O_2$  (H.)*—This substance was mixed with pregnanediol in the first chromatographic separation of the non-ketonic fraction in Case 11. In the second chromatogram it appeared in small amounts with pregnanediol in the fraction removed with 0.3 per cent alcohol in benzene. The fraction that was removed by 0.5 per cent alcohol in benzene, however, was free from pregnanediol. It was best recrystallized from acetone. It melted at 203–205°;  $[\alpha]_D^{28} = -26.3^\circ \pm 1.8^\circ$  ( $c = 0.533$  in alcohol).

$C_{19}H_{32}O_2$ . Calculated, C 77.99, H 11.03; found, C 78.13, H 10.94

The acetate and benzoate of this substance could not be induced to crystallize.

*Isolation of  $C_{19}H_{32}O_2$  (T.)*—In the first chromatogram in Case 5, this substance was eluted from the column along with pregnanediol. The latter was removed as much as possible by crystallization from methanol. The residue was rechromatographed in benzene on a column of alumina. The first nine fractions of 10 ml. each of benzene melted in the range of 185–200°. Nothing more was removed from the column until 0.2 per cent of alcohol was added to the benzene. This solvent removed crystalline material, which melted at 236–237° after recrystallization from methanol and which was identified as pregnanediol.

The first nine fractions were combined and recrystallized twice from acetone. The crystals then melted at 207–209°;  $[\alpha]_D^{27} = +28.1^\circ \pm 1.5^\circ$  ( $c = 0.677$  in alcohol).

$C_{19}H_{32}O_2$ . Calculated, C 77.99, H 11.03; found, C 77.59, H 11.16

Oxidation of 8 mg. of this substance with 0.156 milliequivalent of  $CrO_3$  in 0.7 ml. of acetic acid gave 7 mg. of oil from which a few crystals were

obtained by crystallization from dilute acetone. The crystals melted at 112–113°.

*Substance Possibly Pregnane-3( $\beta$ ), 20( $\alpha$ )-diol*—The fractions in Cases 11 and 13, which were eluted with benzene and benzene with 0.1 per cent of alcohol, on recrystallization from acetone or methanol, yielded plates which melted at 180–182°. The acetate, prepared with pyridine and acetic anhydride at 90°, melted at 142–143°. These properties agree well with those given by Marker and his associates (60) for pregnane-3( $\beta$ ), 20( $\alpha$ )-diol (m.p. 182°) and its diacetate (m.p. 141°). A 3( $\beta$ )-hydroxyl group in the urinary substance was evidenced by the formation of a precipitate with digitonin. Further work must await the accumulation of more material.

*Phenolic Fraction. Isolation of Estrone from Urine in Case 5*—The NaOH washings of the total extract of the hydrolyzed urine were made just acid to litmus and extracted five times with ether. The ether was washed with a solution of sodium carbonate and distilled. The residue, which weighed 2.45 gm., was extracted with a total of 100 ml. of hot benzene. A small aliquot of the benzene solution was removed for bioassay, which indicated the presence of 88,800 rat units of estrogen (equivalent to 177 mg. of estrone). The benzene was washed five times with 100 ml. of 0.3 *M* Na<sub>2</sub>CO<sub>3</sub> to remove "strong" phenols. The benzene was then removed and the residue, in 25 ml. of absolute alcohol, was refluxed 3 hours with 1 gm. of Girard's reagent and 5 ml. of acetic acid. The solution was poured into a mixture of 14.5 ml. of 5 *N* NaOH and ice and extracted three times with ether. The aqueous residue was acidified with 10 ml. of concentrated H<sub>2</sub>SO<sub>4</sub> diluted with ice water. After 3 hours it was extracted with ether. The ether extract was washed with a solution of sodium bicarbonate and distilled. The crystalline residue weighed 140 mg. On recrystallization from 95 per cent alcohol, three crops of crystals, which had a melting point of 247–253°, were obtained for a total weight of 62 mg. Recrystallization from methanol gave large, slightly yellow bars which melted at 255–256°. A mixture with authentic estrone (m.p. 253–255°) melted at 253–255°. The 62 mg. of estrone isolated accounted for approximately 35 per cent of the total estrogenic activity determined by bioassay.

Attempts to isolate estradiol and estriol from the other phenolic fractions proved fruitless. Although a few crystals were obtained from the fraction in which  $\alpha$ -estradiol would be expected, they melted at about 130°, whereas a reference specimen of  $\alpha$ -estradiol, kindly furnished by the Ciba Pharmaceutical Products, Inc., melted at 172–174°.

*Isolation of Glucuronides*—The urine in Case 11 was received in three 18 liter lots which were worked up separately. The concentrated urine (1.5 liters) was extracted seven times with 200 ml. of butyl alcohol. The combined extracts were made slightly alkaline with 5 *N* NaOH and then

were washed three times with 25 ml. of *N* NaOH. The NaOH washings were washed back twice with 50 ml. of butyl alcohol, which was added to the main extract. The butyl alcohol was distilled under reduced pressure and the residue was dissolved in 500 ml. of 0.1 *N* NaOH. This solution was extracted with 200 ml. and three times with 100 ml. of butyl alcohol. The extract was washed twice with 25 ml. of water and taken to dryness. The residue was dissolved in 25 ml. of water, warmed, and 500 ml. of acetone were added. The mixture stood in the refrigerator until the supernatant was clear. The partially crystalline precipitate was dissolved in 100 ml. of 0.1 *N* NaOH and the solution was extracted with 35 ml. and three times with 20 ml. of butyl alcohol. The 0.1 *N* NaOH solution was extracted later, three times with 20 ml. and four times with 50 ml. of butyl alcohol. These extracts separately were washed twice with 10 ml. of water. The residues, after distillation of the butyl alcohol, were dissolved in 3 to 5 ml. of water and 40 to 50 ml. of hot acetone were added slowly with shaking. A crystalline precipitate soon appeared and increased in amount as more acetone was added. A total of 1960 mg. of pigmented crystalline material was thus obtained from 54 liters of urine. It was dissolved in 10 ml. of warm water, 5 ml. of hot acetone were added, and the solution was quickly filtered by suction. Addition of 45 ml. of hot acetone precipitated 468 mg. of white needles which melted at 250–253°. Repeated recrystallizations did not change this melting point or the crystal form. Sodium pregnanediol glucuronide separates as plates under these conditions and melts at about 270°. The mother liquor yielded additional amounts of material.

It was assumed that this material was a mixture of glucuronides. It was dried for 12 hours at 105° and analyzed for glucuronic acid by the method of Maughan, Evelyn, and Browne (66). The intensity of the color produced was 94 per cent of the intensity of color produced by an equal weight of sodium pregnanediol glucuronide.

*Enzymic Hydrolysis of Glucuronides*—The procedure of Talbot, Ryan, and Wolfe (67) was used. 540 mg. of the recrystallized glucuronides were dissolved in 30 ml. of water. 20 ml. of the 0.1 *N* acetate buffer, pH 5.0, 2.5 gm. of the acetone-dried rat liver preparation, and a layer of toluene were added. After incubation at 37° for 48 hours with occasional shaking, the mixture was chilled and filtered. The filtrate and the residue of liver were extracted separately with ether. The ether extracts were combined, washed with NaHCO<sub>3</sub> and water, and distilled. The residue weighed 340 mg. and assay disclosed 84 mg. of 17-ketosteroids. The ketonic material was separated from the non-ketonic with the aid of Girard's reagent. The non-ketonic fraction weighed 234 mg. and the ketonic fraction 80 mg. Chromatographic analysis of the non-ketonic fraction on a 1 by 10 cm. column of alumina gave 47 mg. of pregnane-

3( $\alpha$ ),20( $\alpha$ )-diol and 75 mg. of pregnane-3( $\alpha$ ),17,20-triol, which were identified by comparison of their respective diacetates with authentic specimens. The other fractions (78 mg.) eluted from the column were amorphous. There was an apparent loss of 34 mg. of the non-ketonic fraction.

Four crystalline fractions were obtained by chromatographic analysis of the ketonic fraction, but the amounts were too small to permit identification since their properties did not coincide with any of the known substances.

These results indicate that the crystalline material isolated from the butyl alcohol extract was a mixture of the glucuronidates of pregnane-3( $\alpha$ ),20( $\alpha$ )-diol, pregnane-3( $\alpha$ ),17,20-triol, and various ketones. The pregnanediol and pregnanetriol were present in the ratio 2:3 and the ketones comprised at least 25 per cent of the steroid moiety.

Microanalyses were carried out by A. J. Haagen-Smit, California Institute of Technology, William Saschek, Columbia University, and by one of us (H. L. M.).

#### SUMMARY

Steroids were isolated from the urine of six patients, all women, who had adrenal cortical tumors, and four patients who had bilateral cortical hyperplasia. A new 17-ketosteroid was isolated from the urine of three patients with tumor and all four patients with hyperplasia. It was identified as androstane-3( $\alpha$ ),11-diol-17-one. Two isomeric diols,  $C_{19}H_{32}O_2$ , which melted at 203–205° and 207–209°, were obtained, respectively, from the urine of three patients with hyperplasia and two patients with tumor. From the urine of one patient with a cortical tumor who excreted excessive amounts of estrogens, estrone was isolated in an amount corresponding to approximately 35 per cent of the total estrogenic activity.

The isolation of relatively large amounts of dehydroisoandrosterone from the urine of five of the six patients with cortical tumor and the isolation of only small amounts or none from the urine of patients with hyperplasia confirmed, with one exception, the suggestion of Crooke and Callow (12) that excretion of excessive amounts of dehydroisoandrosterone is characteristic of the presence of an adrenal cortical tumor. Androsterone was isolated from the urine of all four patients with hyperplasia and three patients with tumor. Etiocholan-3( $\alpha$ )-ol-17-one was found much more consistently in the urine of the patients with tumors (five of six cases) than in the urine of those with hyperplasia (one of four cases).

Pregnane-3( $\alpha$ ),20( $\alpha$ )-diol was found in all but one of the ten cases. Pregnane-3( $\beta$ ),20( $\alpha$ )-diol was tentatively identified in two instances by its melting point and that of its acetate. Pregnane-3( $\alpha$ ),17,20-triol was obtained from the urine of three patients with hyperplasia and one with a

tumor.  $\Delta^5$ -Androstene-3( $\beta$ ),16,17-triol was obtained in one case and  $\Delta^5$ -androstene-3( $\beta$ ),17( $\alpha$ )-diol in three cases of tumor.

Pregnane-3( $\alpha$ ),16,17-triol was found to be excreted as a glucuronide which could not be separated from pregnanediol glucuronide by recrystallization of the sodium salts.

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# ESTERIFICATION OF PROTEINS WITH ALCOHOLS OF LOW MOLECULAR WEIGHT

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As part of an investigation of the rôle played by various polar groups in determining the affinity of proteins for water and organic solvents, methods for esterifying the carboxyl groups were sought. Diazomethane, methyl sulfate, and methyl halides not only combine with the carboxyl groups, but also methylate the amino and phenolic groups of proteins (1-4). Reaction with hot methyl-*p*-toluene sulfonate appears to be no more selective and, further, leads to hydrolytic breakdown (5). Treatment with 1,2-epoxides in aqueous solution at room temperature has recently been shown to esterify protein carboxyl groups, but the epoxides also combine with other protein groups under conditions favoring esterification (6). The use of hydrochloric acid as catalyst for the esterification of proteins and polypeptides was suggested by Felix and Reindl (7). However, Kiesel and Znamenskaja (8) showed that the conditions advocated, *i.e.* saturation of the protein suspensions in alcohol with hydrochloric acid gas in the cold, might cause, besides esterification, other reactions such as loss of amide nitrogen and destruction of certain amino acids, particularly of tryptophane.

Freudenberg and Jacob (9) recently reported that acid chlorides were more effective than hydrochloric acid as catalysts for the esterification of organic acids. Various acids were quantitatively transformed to the methyl or ethyl esters by treatment with the respective alcohol and small amounts of acetyl or other acid chlorides for 1 day at room temperature.

This reaction has now been applied to proteins. Several proteins were found to dissolve in cold absolute alcohols within a few minutes to an hour after the addition of catalytic amounts of acetyl chloride. Through protein group analyses, alkoxyl determinations, and experiments with model compounds, it was demonstrated that esterification of the carboxyl groups had occurred. The amino, phenolic, and thiol groups did not react; tryptophane was unaffected.

However, in contrast to the results of Freudenberg and Jacob, equivalent amounts of hydrochloric or sulfuric acid were found to be as effective catalysts for the esterification of both proteins and simple acids as was acetyl chloride. Thus it appears probable that the catalytic action of

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acid chlorides is due to their decomposition by the alcohol with ensuing liberation of hydrochloric acid. Nevertheless it is noteworthy, and not generally recognized, that low concentrations of hydrogen ions (0.02 to 0.10 M) suffice to catalyze complete esterification of many carboxylic acids with methyl alcohol at room temperature in 24 hours. These conditions are considerably milder than those generally employed. They are well suited for use with labile substances, such as proteins.

#### EXPERIMENTAL

The gluten, gliadin, hoof and feather keratin, and crystalline egg albumin and  $\beta$ -lactoglobulin preparations were the same as those used in previous studies (6, 10, 12). Casein was kindly furnished by the Eastern Regional Research Laboratory, the pure, pituitary hormones by C. H. Li, crystalline lysozyme by H. L. Fevold, bovine serum albumin by E. J. Cohn, and zinc insulin crystals by Eli Lilly and Company. Polyglutamic acid was prepared according to Bovarnick (11), with the help of J. C. Lewis; it was characterized in a recent publication (12).

The material to be esterified<sup>1</sup> was suspended in a 20- to 100-fold amount of alcohol. To this was added either acetyl chloride, with cooling, in amounts corresponding to 10 to 20 per cent of the material, or about half as much hydrochloric acid, in methanol or as the 36 per cent aqueous solution. To achieve maximal esterification of proteins, a 100-fold amount of alcohol, brought to 0.1 N concentration by addition of the catalyst, was found favorable. After the mixture had stood at room temperature with occasional shaking for 1 to 6 days, the product was isolated, either by precipitation with ether, by evaporation of the solvent and catalyst, or by lyophilization after dialysis. The same techniques, with the exception of dialysis, were applied in the preparation and isolation of small molecular esters.

With simple acids and polyglutamic acid, the completeness of the reaction could readily be ascertained by titration of the residual acid groups, with phenolphthalein as indicator. The extent of methylation or ethylation of proteins was ascertained by Zeisel analyses as modified by Clark (13). To assure removal of all unbound alcohol, the protein samples were lyophilized from aqueous solution or suspension before analysis. Jansen *et al.* (14) have shown that, with pectin preparations, the last traces of alcohol can be replaced by water more readily than they can be removed by vacuum drying. The various untreated proteins yielded amounts of volatile alkyl groups not exceeding, and often considerably less than, those equivalent to their methionine contents. The methoxyl values in Table II were obtained by subtracting these "blank" values, which presumably were due to reactive S-methyl groups.

<sup>1</sup> Since traces of moisture did not interfere with the reaction, no precautions were taken to dry the materials.

A dye technique (10) was also used for comparing the total acid groups of the original with those of the esterified materials. This technique was found suitable for esters prepared from higher alcohols (butanol, propylene glycol). However, methyl, and, to a decreasing extent, ethyl and *n*-propyl esters were partly hydrolyzed under the test conditions (24 hours in phosphate buffer, pH 11.5).<sup>2</sup> This tendency was decreased when the analyses were performed at 3° instead of at room temperature, but even under such conditions, a completely methylated egg albumin preparation lost about 80 per cent of its methoxyl groups. When shorter time periods were employed for equilibration of the dye with soluble proteins and their methyl esters (1 hour at 3°), the residual acid groups were found somewhat lower in several methylated proteins than in the usual 24 hour test.<sup>3</sup> However, all determinations of the residual acid groups, particularly of the methyl esters of proteins by the dye technique, yielded high values because of the lability of these esters.<sup>4</sup>

The manometric Van Slyke apparatus (16) was used in determining the amino groups of proteins (15 minute reaction period). Free phenolic groups were estimated by the Herriott modification (17) of the Folin method. Since most of the protein derivatives were insoluble at pH 8, all samples were hydrolyzed prior to analysis by refluxing with 6 *N* hydrochloric acid for 6 hours. Control experiments showed that phenolic

<sup>2</sup> Differences in the lability of such esters to alkaline hydrolysis are well recognized (15).

<sup>3</sup> When borate buffer of pH 9.2 was used, the differences between the dye-binding capacities of esterified and original proteins were more marked than those obtained by the usual technique. However, the amounts of dye bound by the untreated proteins under these conditions were much smaller than those bound at pH 11.5, and less than equivalent to their carboxyl groups alone. The lability of the methoxyl groups at 38° and at various pH levels is illustrated by results obtained with methylated egg albumin, the methoxyl content of which fell from 2.4 to 1.6, 1.1, and 0.9 per cent after exposure for 24 hours at pH 8.0, 9.2, and 10.2, respectively.

<sup>4</sup> It may be appropriate to discuss here briefly the limitations encountered during the continued use of the dye techniques for the determination of acid and basic groups in proteins (10). (1) If derivatives of proteins are under investigation, it is essential that any substituents attached to the acid or basic groups be stably bound so that they will not be released under the pH conditions of the test (pH 11.5 and 2.2 respectively). For instance, instabilities at pH 11.5 were encountered with esters of protein carboxyl groups and with acid sulfates of the hydroxyl groups (Reitz, Ferrel, Fraenkel-Conrat, and Olcott, in preparation for press). (2) The pH of the test conditions has to be sufficiently far from the isoelectric point to permit all charged groups of the protein to combine with the oppositely charged dye. This is not the case when the basic groups of pepsin or of sulfated proteins are to be determined (at pH 2.2); it also makes determinations of the acid groups of lysozyme unreliable (see Table II, last foot-note) and of protamine impossible (at pH 11.5). (3) Precipitation of soluble proteins by the dye under the test conditions is prerequisite. Of a great number of proteins tested only isinglass gave no precipitate and the precipitation of some gelatin preparations appeared incomplete.

ethers were stable under these conditions. The Sullivan method (18) was used for comparison of the cysteine contents of methylated and untreated egg albumin and  $\beta$ -lactoglobulin. The proteins were hydrolyzed in evacuated Thunberg tubes. Tryptophane was determined, after alkaline hydrolysis, by the Horn and Jones method (19) with *p*-dimethylaminobenzaldehyde.

### Results

The effectiveness of small amounts of mineral acid (or acetyl chloride) in catalyzing the esterification of macromolecular poly acids at room temperature is well demonstrated with polyglutamic acid. This polypeptide dissolves in methanol, ethanol, and propylene glycol soon after addition of the catalyst. The extent of esterification was ascertained by titration after dialysis (Table I). The acid groups were 97 per cent esterified within 24 hours of treatment with methanol and 0.05 *N* hydrochloric acid. This value was confirmed by methoxyl determinations (calculated for polyglutamic methyl ester, 21.6 per cent  $\text{CH}_3\text{O}$ ; found, 20.8 per cent). The rate of methylation was found to be dependent upon the catalyst concentration. Acetyl chloride, concentrated aqueous hydrochloric acid, and hydrochloric acid in dry methanol were effective in similar molar concentrations. Primary alcohols other than methanol reacted more sluggishly, and isopropanol did not react appreciably. These differences are in agreement with rates of esterification of simple acids (15).

All types of polyglutamic esters were more labile in alkali than were the corresponding protein esters. Thus the most stable ester studied (*i.e.*, that obtained with propylene glycol) was 75 per cent hydrolyzed under the conditions of the dye test (24 hours at 3° in buffer of pH 11.5), while in proteins most of these ester bonds appear to be sufficiently stable to permit the use of the dye technique as a measure of the extent of their occurrence (6).

Several of the proteins investigated resembled polyglutamic acid in dissolving in the alcohols used in the early stages of the reaction. These were serum albumin, insulin, lysozyme,  $\beta$ -lactoglobulin, and the pituitary adrenocorticotrophic and lactogenic hormones. Egg albumin dissolved only in propylene glycol during esterification. The clear alcoholic protein solutions became progressively more viscous or turbid and sometimes set to solid gels within 24 hours, depending upon protein concentration. Other proteins yielded gels without passing through a soluble phase, while others again appeared physically unchanged. White powders could be obtained from the gels or alcoholic solutions by addition of several volumes of ether. Products isolated in this manner were in the form of protein ester hydrochlorides. The alcohol-soluble esters were also readily soluble in water and

dilute salt solutions, but less so in excess acid. Addition of alkali to pH 7 to 8 precipitated these materials and they did not redissolve even when brought to pH 10.5 to 11.0. This solubility behavior is similar to that observed for protein esters prepared by means of epoxides (6). It is attributed to the shift of the isoelectric point to the alkaline side through the blocking of the carboxyl groups in the treated proteins.<sup>5</sup>

It appeared important to ascertain the stability of the peptide and amide bonds of proteins under the conditions used for esterification of their carboxyl groups. Osmotic pressure measurements on polyglutamic acid

TABLE I  
*Esterification of Polyglutamic Acid with Various Alcohols at 22-24°\**

Alcohol	Catalyst	Extent of esterification†		
		4 hrs	1 day	6 days
		per cent	per cent	per cent
Methanol	Hydrochloric acid, 0.05 N‡	64	97	
	“ “ 0.2 “	84		
	Acetyl chloride, 0.05 N	65	97	
Ethanol	Hydrochloric acid, 0.05 N		54	83§
n-Propanol	“ “ 0.05 “			50
Isopropanol	“ “ 0.05 “		0	4
Benzyl alcohol	“ “ 0.05 “			6
Propylene glycol	“ “ 0.05 “		40	76

\* 40 mg. of polyglutamic acid, 0.0001 mole of catalyst, and 2 ml. of alcohol in all but the second experiment, in which 0.5 ml. of methanol was used.

† As determined by titration after dialysis.

‡ Identical results were obtained when hydrochloric acid was added in methyl alcoholic solution, or in 36 per cent aqueous solution.

§ When 5 times as much alcoholic hydrochloric acid solution were used, esterification with ethanol was 90 per cent complete at the end of 6 days. Both the 83 and 90 per cent esterified material were largely insoluble in water, in contrast to the methyl esters of polyglutamic acid.

and its methyl ester, to be reported elsewhere,<sup>6</sup> showed that esterification caused no breakdown in this polypeptide, which is more labile towards acid than most protein peptide bonds. The stability of peptide and amide bonds in acidic alcohol was further indicated by the finding that proteins did not show an increase in the number of their free amino groups or a loss in

<sup>5</sup> The shift of the isoelectric point of a protein to the alkaline side upon methylation was also demonstrated by an electrophoretic study of the methylated lactogenic hormone of the anterior pituitary, performed by Dr. C. H. Li and to be reported by him.

<sup>6</sup> D. K. Mechem, H. S. Olcott, and H. Fraenkel-Conrat, in preparation for press.

amide groups after methylation.<sup>7</sup> The following observation also may be regarded as evidence for the integrity of the protein molecule under the conditions of methylation. A partially methylated bovine serum albumin preparation (4.5 methoxyl groups per  $10^4$  gm. of protein) yielded a small fraction which remained water-soluble after electrodialysis. This fraction was heat-coagulable (at  $70^\circ$ ) and therefore possessed at least one property of a "native" protein.

Representative data concerning the maximal extent of methylation of various proteins at room temperature and of the presence of 0.1 N hydrochloric acid are compared, in Table II, with their content of total acid and carboxyl equivalents. The latter were computed by subtracting the number of phenolic and sulfhydryl groups from the total acid groups. The agreement in most cases between the carboxyl contents of the untreated proteins and the methoxyl contents of the methylated proteins is suggestive evidence for the specificity of the reaction for carboxyl groups.

In many experiments, not listed in detail, the effect of variations in the experimental conditions on the extent of methylation was investigated. The same extent of methylation was obtained with equivalent amounts of acetyl chloride and hydrochloric or sulfuric acid as catalyst. With proteins, in contrast to simple or polymeric acids, the amount of catalyst used appeared of greater importance than the catalyst concentration. Thus at least 30 to 50 equivalents of acid (or acid chloride) had to be used per  $10^4$  gm. of protein in order to approach complete methylation of the carboxyl groups. Under these conditions, about 75 per cent of these groups had reacted within 1 day, while with less catalyst (10 equivalents) only 25 per cent of the carboxyl groups was methylated in 1 week. The concentration of the catalyst in all these experiments was 0.05 or 0.1 M. When ethanol was used under conditions favoring maximal esterification, fewer equivalents were introduced into egg albumin than with methanol (*cf.* foot-note to Table II).

A few experiments were performed at elevated temperatures ( $53^\circ$  and  $70^\circ$ ). The amounts of methoxyl introduced into several proteins were then

<sup>7</sup> Dialyzed preparations were used for amide analyses, since isolation by precipitation and washing with ether might not remove ammonium chloride if it were formed during the reaction. The presence of ammonium chloride in their preparation may explain the erroneously high values of Felix and Reindl (7) for the acid and basic groups of gliadin as determined by the methoxyl and chloride contents after esterification in methyl alcohol saturated with hydrochloric acid. However, in an attempt to repeat their experiments, although the gliadin dissolved as reported by them, the isolated product contained only 3.8 methoxyl groups per  $10^4$  gm. of protein in contrast to the value of 19.5 groups found by Felix and Reindl. No appreciable amounts of ammonium chloride could be detected. The value of 3.8 groups agrees with the calculated number of carboxyl groups (see Table II).

in excess of their original carboxyl groups. The products differed from protein esters prepared at room temperature in having increased amino and decreased amide nitrogen contents, evidence that some methanolysis

TABLE II

*Reaction of Proteins with Methyl Alcohol at 22-24° in Presence of Small Amounts of Mineral Acid or Acetyl Chloride\**

Protein	Groups per 10 <sup>4</sup> gm. proteins†			Groups in methylated proteins, ‡ per cent of those of original proteins	
	Original total acid	Original carboxyl:	Introduced methoxyl	Phenol + indole	Amino
$\beta$ -Lactoglobulin.....	16.9	13.7‡	13.6	101	98
Serum albumin (bovine).....	14.3	10.8	10.3	94	
Casein.....	19.3	16.0§	10.0	98	104
Egg albumin.....	12.7	9.5	9.0	100	88
Insulin.....	14.6	8.0	8.4	100	96
Lactogenic hormone.....	11.8	8.8	7.9	100	
Hoof powder.....	9.5	6.6	6.2		97
Lysozyme¶.....			4.2	97	100
Gluten.....	5.4	3.6	3.6		
Silk fibroin.....	8.6	2.4	2.3	101	
Gliadin.....	5.4	3.9	2.1		100
Deamidated gluten (12).....	27.7		18.2		

\* 30 to 50 equivalents of acid or acetyl chloride per 10<sup>4</sup> gm. of protein, in 0.02 to 0.1 N solution; reaction period, 2 to 6 days.

† Values not corrected for moisture. The moisture content of various proteins equilibrated to laboratory conditions varied from about 6 to 10 per cent and was not appreciably affected by methylation. The total acid groups were determined according to Fraenkel-Conrat and Cooper (10).

‡ These values were calculated by subtracting the number of tyrosine and sulfhydryl groups from the total acid groups. The values are in general agreement with those obtained by other authors through direct analyses, as summarized by Fraenkel-Conrat and Cooper (10).

§ The phosphoric acid groups of casein (2.7 equivalents of phosphorus per 10<sup>4</sup> gm.) would be included in this value. They probably are not as readily methylated as the carboxyl groups.

|| When ethyl alcohol was used, 6.0 equivalents were introduced in 5 days.

¶ The strongly basic nature of this protein prevented a reliable determination of its acid groups by the dye technique (see foot-note 4).

occurred at the elevated temperatures. However, proteins that did not dissolve during the reaction at room temperature remained largely insoluble also at 53° and 70°.

The extent of esterification of proteins with alcohols other than methanol was estimated by the dye technique. Representative data on the extent of

reaction with egg albumin show that only partial esterification was achieved (Table III). Like polyglutamic acid, proteins react less readily with

TABLE III  
*Esterification of Egg Albumin with Alcohols Other Than Methanol\**

Alcohol	Reduction in carboxyl groups†
	<i>per cent</i>
Ethanol... ..	50‡
n-Propanol.....	50
Isopropanol... ..	5
n-Butanol... ..	26
Propylene glycol . . . . .	28

\* 1 gm. of air-dry protein in 100 ml. of the respective alcohol, containing 1 ml. of concentrated hydrochloric acid, permitted to stand at room temperature for 5 days. Only reaction with propylene glycol led to solution of the protein.

† Calculations based on determinations of total acid groups by the dye technique (10) at 3°. Analyses at room temperature indicated a lesser extent of esterification in the case of ethanol and propanol (15 and 33 per cent).

‡ Alkoxy determinations indicated esterification of 63 per cent of the carboxyl groups in this sample.

TABLE IV  
*Methylation of Acids at Room Temperature in Presence of 0.1 Equivalent of Hydrochloric Acid*

Acid*	Extent of esterification after	
	24 hrs.	48 hrs.
	<i>per cent</i>	<i>per cent</i>
Acetic acid	99	
Lactic " .. .	99	
Benzoic acid†	6	11
Galaeturonie acid . . . . .	86	100
Thioglycolic acid‡	6	
Benzoyl-dl-alanine . . . . .	97	100
Acetyl-dl-tryptophane . . . . .	91	99
N-Benzoyl-dl-serine ... ..	68	93

\* Concentration of organic acid 0.3 N, of hydrochloric acid 0.03 N.

† 26 per cent esterification after 6 days. When 0.43 equivalent of hydrochloric acid was used, in 0.32 M solution, 44 per cent esterification occurred within 24 hours and 77 per cent within 4 days.

‡ 20 per cent esterification after 10 days. Acetic acid was readily methylated in the presence of thioglycol, thus indicating that thiols do not inhibit the esterification of fatty acids.

higher primary alcohols than with methanol, and they do not react appreciably with the secondary alcohol, isopropanol.

There were no significant differences between the amino nitrogen of methylated and untreated proteins (Table II). Similarly, the phenolic groups were found free in the treated proteins (Table II). Neither the thiol nor indole groups were affected by the reaction. Thus methylated egg albumin and  $\beta$ -lactoglobulin contained 90 to 100 per cent of the cysteine and tryptophane present in the original proteins. Some or all of these types of protein groups are affected by other reagents for esterification, such as epoxides, diazo compounds, methyl sulfate, or halides (1-6), or by the use of excessive amounts of acid as catalyst (7, 8).

The applicability of the esterification method with catalytic amounts of mineral acid was also studied with a few simple compounds.<sup>8</sup> Marked differences in reactivity, conforming in general with their behavior in other methods of esterification (15), were observed (Table IV). The claim of Freudenberg and Jacob (9) that hydrochloric acid did not catalyze the formation of methyl stearate as effectively as did acetyl chloride could not be confirmed. Methyl stearate of similar purity was obtained with equivalent amounts of either catalyst during 24 hours at 22-25°.<sup>9</sup>

To study the specificity of the method, various phenols and mercaptans were treated with methyl alcohol under conditions favoring rapid and quantitative esterification of carboxylic acids. There was no measurable decrease in the phenolic and thiol groups present in the reaction mixtures, as determined by the Folin reactions. After *p*-cresol had been treated with 0.1 *N* hydrochloric acid in methanol for 5 days, its chromogenic value (phenol reagent) was 98.5 per cent of that of the control solution containing no acid. Under similar conditions, thioglycol retained its sulfhydryl group quantitatively as measured with Folin's uric acid reagent. Thus the model experiments with simple compounds confirm the observation that, of the types of groups which occur in proteins, apparently only the carboxyl groups are involved in the reaction.<sup>10</sup>

#### SUMMARY

Carboxyl groups of proteins and model substances are readily methylated at room temperature in methyl alcohol containing small amounts of mineral acids (0.02 to 0.1 *N*). The catalytic activity of acid chlorides in esterifications is due to the liberation of hydrochloric acid. The acid-catalyzed

<sup>8</sup> In contrast to acylamino acids, free amino acids had to be treated with at least 1.1 equivalents of acid to achieve methylation at room temperature.

<sup>9</sup> Freudenberg and Jacob (9) used 20° for their experiments.

<sup>10</sup> E. F. Jansen of this Laboratory has observed some methyl glucoside formation in conjunction with the esterification of galacturonic acid by the present technique (in preparation for press). While the rate of the latter reaction greatly exceeded that of the former, the possibility must be recognized that any polysaccharides containing reducing groups, if such occur in proteins, may participate in the methylation reaction.



reaction of proteins with methanol appears to be a specific one involving only the carboxyl groups; amino, phenolic, thiol, and indole groups and peptide and amide bonds are unaffected. Esterification occurs also with higher primary alcohols, but is slower and progressively less complete than with methanol. Several proteins dissolve in the alcohols during the reaction.

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# THE ISOLATION OF PREGNANEDIOL-3 $\alpha$ ,17-ONE-20 FROM HUMAN URINE\*

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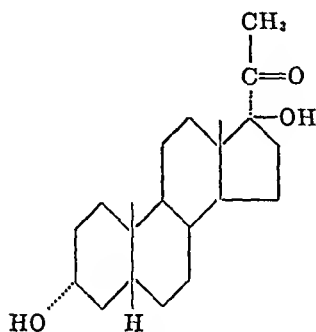
Qualitative and quantitative results obtained in the course of a systematic investigation of the ketosteroids present in the urine of normal persons, of pregnant women, and of individuals with leucemia, cancer, and disorders of the adrenal gland have been described in previous communications (1) from this laboratory. In the present report is given a description of a hitherto unknown ketosteroid, pregnanediol-3 $\alpha$ ,17-one-20 (I), which has been isolated from several different urine specimens.

The compound is a diastereoisomer of allopregnanediol-3 $\beta$ ,17 $\beta$ -one-20 (II), which was isolated from beef adrenals by Wintersteiner and Pfiffner (2), who called it Compound G, and by Reichstein (3), who called it Compound L. Likewise it is closely related to Compounds J (III) and O (allopregnanetriol-3 $\beta$ ,17 $\beta$ ,20 $\beta$  and its 20-epimer) which Reichstein (3, 4) isolated from beef adrenals, and also to pregnanetriol-3 $\alpha$ ,17,20 (IV), which Butler and Marrian (5) isolated from the urine of two women with adrenal tumors. The presence of a tertiary hydroxyl group on C-17 is a distinctive characteristic of these and many other substances which have been isolated from adrenal tissue. For this reason, it is highly probable that pregnanediol-3 $\alpha$ ,17-one-20 is derived from steroids of adrenal origin. The immediate precursor of this compound is uncertain, but it may be 17-hydroxyprogesterone (V), which has been isolated from beef adrenals (6) or perhaps one or the other of the adrenal steroids having ketol side chains. The precursor might even be a substance possessing a dihydroxyacetone side chain, like Reichstein's Compound S ( $\Delta^5$ -pregnenediol-17 $\beta$ ,21-dione-3,20) (7). This possibility is supported by the observations (8-10) that desoxycorticosterone acetate is metabolized in both animals and humans to pregnanediol, an indication that the organism is capable of reducing the CH<sub>2</sub>OH group at the 21 position to a methyl group.

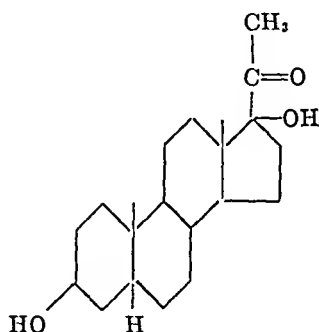
Careful examination of very large amounts of steroidal material in

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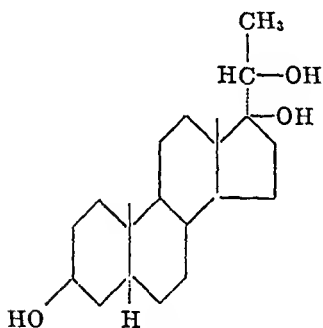
the urine of normal individuals or pregnant women has failed to reveal the presence of pregnanediol-3 $\alpha$ ,17-one-20. It seems justifiable, therefore, to assume that the substance is a product of either an abnormal activity of the adrenal gland or an abnormal metabolism of steroids of adrenal origin. Possibly it is an intermediate reduction product between adrenal steroids and the urinary pregnanetriol (IV) which also has been



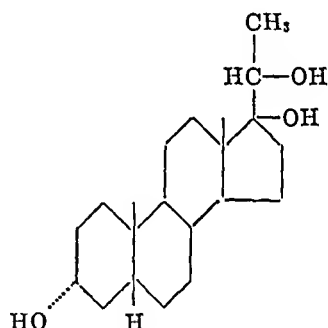
(I)



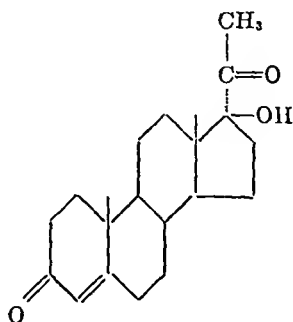
(II)



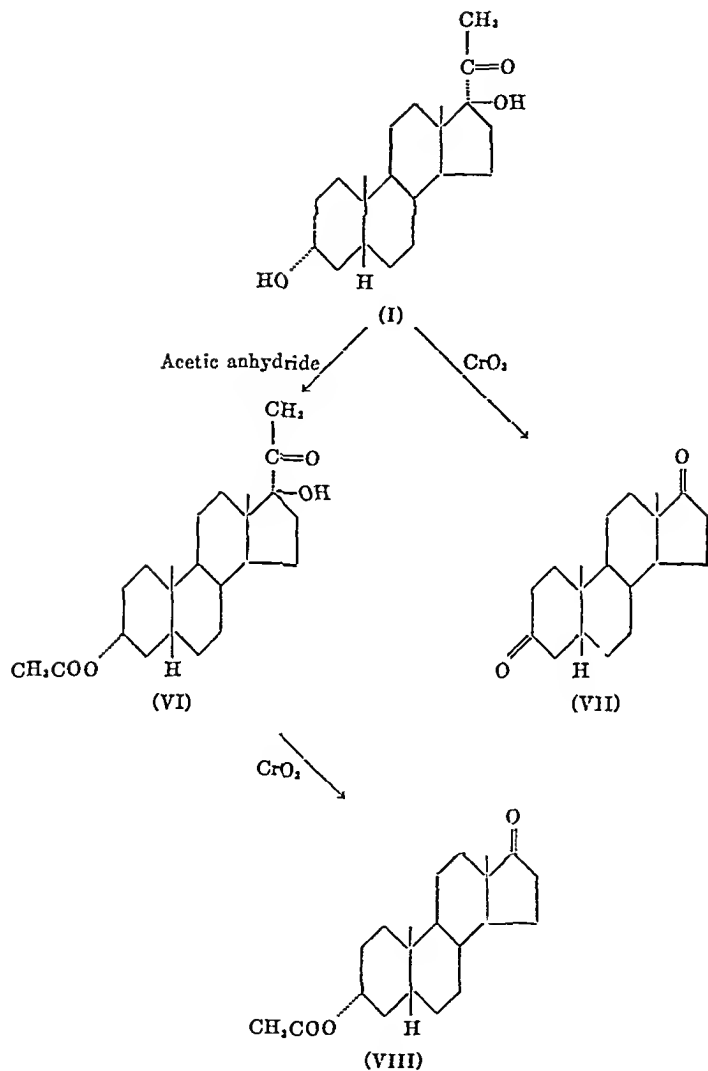
(III)



(IV)



(V)



isolated only from the urine of individuals with abnormalities of the adrenals. To obtain further evidence concerning this point, the non-ketonic alcoholic fractions from the urine specimens containing pregnane-

diol-3 $\alpha$ ,17-one-20 are now being examined for the presence of the pregnanetriol.

About 70 mg. of the new steroid have been obtained from 22 liters of urine excreted by a woman having hyperplasia of the adrenal gland,<sup>1</sup> about 700 mg. from 121 liters (about 128 days) of urine of a cryptorchid male,<sup>2</sup> 8 mg. from 17 liters (19 days) of urine of a woman with an adrenal tumor,<sup>1</sup> and 1 mg. from 141 liters (105 days) of urine of a eunuchoid male given testosterone by injection.<sup>2</sup>

The evidence follows for the conclusion that the compound obtained is pregnanediol-3 $\alpha$ ,17-one-20. The substance was separated from the other constituents of the ketonic  $\alpha$ -steroid fraction of urine by chromatographic analysis on alumina or on a mixture of magnesium silicate and Celite. It was eluted from the alumina with a solution of 30 per cent acetone in ether and from the mixture with a solution of 40 per cent benzene in carbon tetrachloride. Crystallization of these eluates from acetone produced beautiful needles which melted at 210–212°. Further crystallization raised the melting points of some samples to 219–219.5°. The specific rotation of the material in ethanol was  $[\alpha]_D^{28} = -7.8^\circ \pm 2.6^\circ$ .

Elementary analysis gave the empirical formula  $C_{21}H_{34}O_3$ , which indicates a pregnane derivative possessing two hydroxyl groups and one carbonyl group. The compound reacted with acetic anhydride in pyridine to form a monoacetate (VI), indicating that the unreactive hydroxyl group is either on C-11 or in a tertiary position. The substance reacted with hydroxylamine to form a monoxime. Oxidation with chromium trioxide in acetic acid yielded a crystalline product melting at 132–134° which was identical with etiocholanedione-3,17 (VII), thereby proving that the compound is a steroid of the etiocholane series (H on C-5 *cis* to methyl on C-10). Furthermore, one oxygen substituent is on C-3 and the remaining two must be present in the side chain, so arranged that degradation by chromium trioxide oxidation results in a product ( $C_{19}H_{28}O_2$ ) (VII) having 2 less carbon atoms than the original compound and having a carbonyl group at the 17 position. These considerations, together with the fact that one of the two hydroxyl groups in the molecule was not acetylated when treated with acetic anhydride, indicate the presence of a tertiary hydroxyl group on C-17 with the third oxygen substituent vicinal to it on C-20. Only such a structure could yield on chromium trioxide oxidation etiocholanedione-3,17. Oxidation of the monoacetate (VI) with chromium trioxide produced the acetate of etiocholanol-3 $\alpha$ -one-17 (VIII), demon-

<sup>1</sup> Obtained through the courtesy of Dr. Fuller Allbright, Massachusetts General Hospital, Boston.

<sup>2</sup> Obtained through the courtesy of Dr. Ephraim Shorr, the New York Hospital, New York.

strating that the hydroxyl group which had been acetylated is of the  $\alpha$  configuration on C-3 and therefore the reactive carbonyl group must be on C-20. Consequently the structure of this new urinary steroid is that of the hitherto unknown pregnanediol-3 $\alpha$ ,17-one-20 (I) or 17-hydroxypregnanolone. Since Reichstein *et al.* (11, 12) have demonstrated that all the cortical steroids having a hydroxyl group on C-17 are of the same steric configuration at that asymmetric center, it is probable that the 17-hydroxyl group in pregnanediol-3 $\alpha$ ,17-one-20 is in the  $\beta$  position. This substance does not reduce Tollens' reagent, nor is it oxidized by periodic acid. Von Euw and Reichstein (13) have demonstrated that Compound L (II) with a ketol side chain like pregnanediol-3 $\alpha$ ,17-one-20, also is resistant to oxidation by periodic acid.

#### EXPERIMENTAL<sup>3, 4</sup>

The urine from the cryptorchid male was acidified to pH 1 with sulfuric acid and extracted continuously with ether for 48 hours. The acidified urine was then refluxed for 30 minutes and reextracted with ether for 24 hours. These two ether extracts were processed as described below, and from each pregnanediol-3 $\alpha$ ,17-one-20 was isolated. All the other urine specimens were hydrolyzed by boiling for 30 minutes at pH 1 and then extracted continuously with ether.

The ether extracts were divided into their neutral, phenolic, and acidic components. The ketonic and non-ketonic components of the neutral fractions were then separated from each other by means of Girard's reagent. The ketonic materials were divided by means of digitonin into the ketonic  $\alpha$ - and  $\beta$ -steroids. The  $\alpha$ -ketonic fractions so obtained were chromatographed either on aluminum oxide (Merck) or on a mixture of magnesium silicate and Celite (2:1).

A complete detailed description of the chromatographic methods employed in the separation of the ketosteroids described in this and in the previous reports (1) will be given in a subsequent paper. Here the techniques are illustrated by an outline of the adsorption procedure used to separate pregnanediol-3 $\alpha$ ,17-one-20 from the other ketosteroids found in the urine of the cryptorchid male. Because the complete separation of a complex mixture of ketosteroids cannot be achieved with a single chromatogram, a systematic procedure modeled after that used in fractional crystallization was developed. This procedure is best illustrated by the following example.

A portion of the ketonic  $\alpha$ -steroid fraction (representing a 58 day specimen) of the acidified (but not boiled) urine of the cryptorchid male

<sup>3</sup> The microanalyses reported herein were done by the courtesy of Dr. A. Elek, The Rockefeller Institute for Medical Research, New York.

<sup>4</sup> All melting points are corrected.

contained 990 mg. of ketosteroid as measured by the Zimmermann colorimetric method (14). This was dissolved in 300 cc. of carbon tetrachloride, and the solution poured through a column (diameter, 55 mm.) of 55 gm. of magnesium silicate-Celite (2:1). A series of solvents was passed through the column so that the adsorbed material was separated into five fractions. The fractions, their eluants, and their ketosteroid contents<sup>5</sup> are shown in Table I.

Fractions AI and AII were combined and rechromatographed, but since no pregnanediol-3 $\alpha$ ,17-one-20 was isolated from these fractions, they will not be described further. Fractions AIV and AV (90 mg.) were combined and submitted to another Girard separation in order to remove the non-

TABLE I

*Chromatogram A, Ketonic  $\alpha$ -Steroid Fraction from Urine of Cryptorchid Male*

Fraction	Volume	Eluant	Amount recovered*	Remarks
	cc.		mg.	
AI	900	Carbon tetrachloride	236	
AII	500	" " + 25% benzene	137	
AI	500	" " + 50% "	425	Rechromatographed in C
	500	Benzene		
	500	" + 25% ether		
AIV	500	" + 50% "	43	Rechromatographed in B
	250	Ether		
AV	500	" + 50% methanol	47	" "
	250	Methanol		
	250	Glacial acetic acid		
Total.....			888	

\* Measured colorimetrically.

ketonic impurities still present. The introduction of Girard separations before each of the subsequent chromatograms was found to be extremely valuable, because the subsequent separations were thereby rendered more efficient. As a result, the number of chromatograms necessary to effect the complete separation of the ketosteroids was reduced by one-half.

The ketonic fraction so obtained (87 mg.)<sup>5</sup> was dissolved in 100 cc. of carbon tetrachloride and poured into a column (diameter, 25 mm.) of 7 gm. of magnesium silicate and Celite (2:1). The development of the column and the fractions obtained are shown in Table II.

Only one additional chromatogram was necessary in order to obtain

<sup>5</sup> Measured colorimetrically.

crystalline pregnanediol-3 $\alpha$ ,17-one-20. Fractions AIII (425 mg.) and BI (30 mg.) were combined and submitted to another Girard separation. The ketonic fraction (450 mg.)<sup>s</sup> was dissolved in 200 cc. of carbon tetrachloride and poured into a column (diameter, 55 mm.) of 40 gm. of magnesium silicate-Celite (2:1). The chromatogram is outlined in Table III.

Recrystallization of Fraction CII from acetone yielded about 100 mg. (by weight) of pregnanediol-3 $\alpha$ ,17-one-20, m.p. 209–212°.

TABLE II  
*Chromatogram B, Fractions AIV and AV*

Fraction	Volume	Eluant	Amount recovered*	Remarks
BI	200	Carbon tetrachloride + 25% benzene	30	Rechromatographed in C
	200	" " + 50% "		
	300	Benzene		
	200	" + 10% ether		
	200	" + 20% "		
	200	" + 40% "		
	200	" + 50% "		
BII	100	Ether	27	
	200	" + 2% methanol		
	200	" + 5% "		
	200	" + 10% "		
BIII	200	" + 20% "	7	
	200	" + 30% "		
BIV	200	" + 40% "	11	
	200	" + 50% "		
	100	Methanol		
	100	Glacial acetic acid		
Total			75	

\* Measured colorimetrically.

From another portion of acidified (but not boiled) urine (70 days) of this same cryptorchid male the  $\alpha$ -ketonic fraction was submitted to a similar series of fractional chromatograms and an additional 160 mg. of the new steroid were obtained thereby. The  $\alpha$ -ketosteroids obtained from the acidified (128 days) urine which had been refluxed for 30 minutes were processed similarly and yielded 440 mg. of crystalline steroid.

The  $\alpha$ -ketosteroids obtained from the urine of the woman with an adrenal tumor and from the urine of the eunuchoid male were chromatographed on a mixture of magnesium silicate and Celite and separated as



described above. The  $\alpha$ -ketosteroids obtained from the urine of the woman having hyperplasia of the adrenal gland were chromatographed on aluminum oxide (Merck). After a similar fractional analysis involving five chromatograms crystalline pregnanediol-3 $\alpha$ ,17-one-20 was eluted by a solution of 30 per cent acetone in ether.

The crystalline eluates from the chromatograms were recrystallized from methanol, from a mixture of benzene and ligroin, or best from acetone, to give flat needles melting at 210–212°. Further recrystallization raised the melting point to 219–219.5°. The specific rotation is  $[\alpha]_D^{28} = -7.8^\circ$

TABLE III  
*Chromatogram C, Fractions AIII and BI*

Fraction	Volume	Eluant	Amount recovered*	Remarks
	cc.		mg.	
CI	800	Carbon tetrachloride	185	
CII	1400	" " + 10% benzene	203	Crystalline pregnanediol-3 $\alpha$ ,17-one-20
	1000	" " + 20% "		
	600	" " + 40% "		
	400	" " + 50% "		
	600	Benzene		
	600	" + 10% ether		Non-crystalline residue
CIII	600	" + 20% "	2	
	600	" + 30% "		
CIV	400	" + 40% "	47	
	400	" + 50% "		
	200	Ether		
	400	" + 10% methanol		
	400	" + 50% "		
	200	Methanol		
	200	Glacial acetic acid		
Total.....			437	

\* Measured colorimetrically.

$\pm 2.6^\circ$  ( $c = 0.379$  in ethanol). When 1 mg. was assayed by the modified Zimmermann colorimetric method (14), a value equivalent to 0.14 mg. of androsterone was obtained.

*Analysis*— $C_{21}H_{34}O_3$ . Calculated. C 75.40, H 10.29  
 Found. " 75.26, " 10.39  
 " 75.33, " 10.52

*Monoacetate*—30 mg. of the compound were refluxed for 1 hour in 1 cc. of pyridine containing 0.5 cc. of acetic anhydride. On dilution with water

a crystalline product was obtained which, when recrystallized from a mixture of acetone and ligroin, formed rosettes melting at 201–202°. This compound was eluted from alumina with a solution of 25 per cent ether in benzene.

*Analysis*— $C_{22}H_{34}O_4$ . Calculated, C 73.36, H 9.64; found, C 73.29, H 9.60

*Monoxime*—20 mg. of the compound were refluxed for 4 hours in 2 cc. of an 80 per cent methanol solution containing 50 mg. of hydroxylamine hydrochloride and 50 mg. of sodium acetate. Dilution with water precipitated 21 mg. of a product which melted at 244–246° (with decomposition). Recrystallization from aqueous methanol gave needles which decomposed at 247–250°.

*Analysis*— $C_{21}H_{31}O_3N$ . Calculated. C 72.16, H 10.10, N 4.01  
Found. " 72.11, " 10.17, " 4.22

*Oxidation of Pregnanediol-3 $\alpha$ ,17-one-20 with Chromium Trioxide*—50 mg. of the substance were dissolved in 0.5 cc. of glacial acetic acid. To the solution were added 2 cc. of a 2 per cent chromium trioxide solution in 90 per cent acetic acid and the mixture was allowed to stand at room temperature for 22 hours. After dilution with water and extraction with ethyl acetate a neutral residue was obtained weighing 40 mg. It was dissolved in 100 cc. of a mixture of ligroin and benzene (1:1) and poured through a column of alumina (5  $\times$  1.5 cm.). Developing the column with the same solvent mixture yielded in the eighth, ninth, and tenth 100 cc. portions 18 mg. of a crystalline residue which when recrystallized from ligroin (b.p. 30–50°) melted at 132–134°. When mixed with an authentic sample of etiocholanedione-3,17 melting at 131–132°, the mixture melted at 131–132°. A sample of this diketone was prepared for analysis by subliming in a high vacuum ( $10^{-5}$  mm. of Hg).

*Analysis*— $C_{31}H_{42}O_2$ . Calculated, C 79.12, H 9.78; found, C 79.02, H 10.00

2 mg. of the crystalline eluate were refluxed in 0.5 cc. of methanol containing 5 mg. of dinitrophenylhydrazine and 1 drop of concentrated HCl. In a few minutes a yellow derivative precipitated. It melted at 263–264° and did not depress the melting point of the bisdinitrophenylhydrazone made from etiocholanedione (calculated for  $C_{31}H_{42}O_2N_8$ , N 17.28 per cent; found, N 17.00 per cent).

*Oxidation of Monoacetate with Chromium Trioxide*—To a solution of 42 mg. of the acetate in 0.6 cc. of glacial acetic acid, 0.8 cc. of a 2 per cent chromium trioxide solution in 90 per cent acetic acid was added. After the mixture had stood at room temperature for 5 hours, water was added and the solution was extracted with ethyl acetate. The neutral oily

residue so obtained weighed 40 mg. It was dissolved in 100 cc. of ligroin and chromatographed on a column of alumina ( $7 \times 1.5$  cm.). 15 mg. of an oily product were eluted by a mixture of ligroin and benzene (1:1). It was dissolved in ligroin and after standing overnight crystallized as rosettes melting at 92–96°. Recrystallization from ligroin raised the melting point to 96–97°. This product did not depress the melting point of an authentic sample of etiocholanol-3 $\alpha$ -one-17 acetate, m.p. 94–95°.

#### SUMMARY

A new steroid, C<sub>21</sub>H<sub>34</sub>O<sub>3</sub>, m.p. 219–219.5°, has been obtained from the urine of a woman with adrenal hyperplasia, of a cryptorchid male, of a woman with an adrenal tumor, and of a eunuchoid male given testosterone by injection. It formed a monoacetate and a monoxime, and yielded on chromium trioxide oxidation etiocholanedione-3,17. The monoacetate was oxidized by the same oxidant to etiocholanol-3 $\alpha$ -one-20 acetate. Therefore the new urinary steroid is pregnanediol-3 $\alpha$ ,17-one-20.

Because of the presence of a tertiary hydroxyl group on C-17, a characteristic of some of the steroids produced by the adrenal gland, there can be little doubt that the compound described is of adrenal origin.

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# THE METABOLISM IN VIVO OF *dl*-PHENYLALANINE IN THIAMINE DEFICIENCY\*

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It is well established that phenylalanine and tyrosine are incompletely metabolized in scorbutic guinea pigs (15-17) and by premature infants (9, 10). Closs and Fölling (3) have reported that phenylpyruvic acid appeared in the urine of thiamine-deficient rats when extra phenylalanine was fed. The evidence in support of the latter claim was not conclusive, but such a finding would not be inconsistent with the abnormally great excretion of pyruvic acid in thiamine deficiency (14, 8) and the rôle of thiamine in the decarboxylation of both  $\alpha$ -ketomonocarboxylic acids (11) and  $\alpha$ -ketoglutarate (1, 7). Furthermore, there is some evidence to suggest that ascorbic acid synthesis by the rat and the dog is dependent upon an adequate supply of thiamine (19, 6, 4). Hence, it is conceivable that thiamine deficiency could lead to a secondary ascorbic acid deficiency in these species and, thereby, explain such findings as reported by Closs and Fölling.

In efforts to develop methods for the detection of vitamin deficiencies in man based upon the appearance of metabolic defects arising from the deficiency it is important to know whether the metabolic defect is specific for the condition under test. The question arises, therefore, whether thiamine lack as well as ascorbic acid deficiency might give rise to the appearance in urine of products of the incomplete metabolism of phenylalanine. The experiments here reported were carried out to investigate this point.

## Methods

The thiamine-deficient diet used was of the following composition: vitamin-free casein (S. M. A. Corporation) 25, sucrose 64, cottonseed oil 8, and salt mixture (Osborne and Mendel (13)) 3, plus 5 mg. of riboflavin, 30 mg. of calcium pantothenate, 2.5 mg. of pyridoxine, 0.5 gm. of choline chloride, and 1 gm. of inositol per kilo of diet. Twice a week each rat received 2 drops of a mixture containing equal parts of cod liver oil and wheat germ oil. The control diet contained in addition 2.5 mg. of thiamine

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per kilo of diet. The animals were kept in individual wire mesh cages and transferred to individual metabolism cages during the periods when urine was collected.

The urines were analyzed for total hydroxyphenyl compounds (12) by the Millon reaction as modified by Folin and Ciocalteu (5) and adapted for use with the Evelyn photoelectric colorimeter. After phenylpyruvic acid and other ether-soluble compounds had been extracted from the acid urine in the cold by shaking with an equal volume of ethyl ether, the non-extractable hydroxyphenyl compounds were determined by repeating the same procedure. The results are expressed as "tyrosine equivalents," with no attempt to define the exact products responsible for the reaction.

The reduction of phosphomolybdic acid by the urines was taken as an estimate of aromatic keto acid excretion (18, 12, 10). This reduction was carried out by the Bodansky method for phosphorus (2) but with omission of the stannous chloride and addition of  $M/15 \text{ KH}_2\text{PO}_4$ . The intensity of the color was read in the Evelyn photoelectric colorimeter at the end of 3 hours and compared with a phosphorus standard which was prepared in the usual manner. No attempt was made to relate the values so obtained to the actual content of keto acids, as the interest was only in the comparative figures. The results are expressed as "phosphorus equivalents." Each urine was also tested qualitatively with ferric chloride, which reacts with either phenylpyruvic acid (green) or homogentisic acid (blue).

#### EXPERIMENTAL

Albino rats, four females and one male, of the Fischer strain 4 weeks of age and weighing 36 to 53 gm. were placed alternately on the control diet (3 weeks), the thiamine-free diet (5 weeks), the control diet (4 weeks), and, again, the thiamine-free diet (8 weeks). All of the rats exhibited signs of thiamine deficiency such as marked loss of weight, typical hunched posture, and, in some cases, spasticity and paralysis while on the deficient régime, but they developed well during the control feeding. Two of the animals died during the period of deficiency.

During each period a number of 24 hour urine samples were collected in bottles containing 1 cc. of glacial acetic acid. The urine was preserved by deep freezing ( $-20^\circ$ ) for 1 to 5 days until the analyses were made. At intervals a solution of 100 or 200 mg. of *dl*-phenylalanine (Merck) dissolved in water with an equivalent amount of sodium carbonate was administered by stomach tube, and the urine was collected in the manner described during the subsequent 24 hour period.

The urinary excretion values during the various periods are summarized in Table I. The *t* test applied to these data reveals that the differences between the mean values for total phenols and for reducing substances

(phosphorus equivalent) for the first two experimental periods were statistically significant ( $P < 0.05$ ). The findings suggest that more phenylpyruvic acid was excreted by the rats on the control diet than when they were deficient in thiamine. An increase of excretion of hydroxyphenyl compounds was again noted when the rats were returned to the control diet (Period III). Less marked differences were observed between the second control period and the following deficiency period. At no time did any of these urines give a color with ferric chloride.

TABLE I

*Analyses of 24 Hour Urine Specimens of Rats Fed Alternately Diets with and without Thiamine*

Period No.	Diet	No. of animals	No. of urine specimens	Hydroxyphenyl compounds as tyrosine				"Phosphorus equivalent"	
				Before ether extraction		After ether extraction			
				Range	Mean	Range	Mean	Range	Mean
I	Control	5	10	mg. 2.5-3.5	mg. 2.90	mg. 1.5-3.3	mg. 2.52	0.017-0.073	0.0362
II	Thiamine-free	5	13	1.1-3.1	2.03	1.2-3.2	2.05	0.003-0.021	0.0132
III	Control	3	6	4.0-7.4	5.27	3.5-6.3	4.60	0.014-0.029	0.0232
IV	Thiamine-free	3	9	3.2-6.4	4.54	3.0-4.6	3.63	0.020-0.033	0.0271

TABLE II

*Analyses of 24 Hour Urine Specimens of Rats Fed Alternately Diets with and without Thiamine following Single Doses of 100 Mg. of Phenylalanine*

Period No.	Diet	No. of animals	No. of urine specimens	Hydroxyphenyl compounds as tyrosine				"Phosphorus equivalent"	
				Before ether extraction		After ether extraction			
				Range	Mean	Range	Mean	Range	Mean
II	Thiamine-free	3	3	mg.	mg.	mg.	mg.	mg.	mg.
III	Control	3	6	1.8-2.8	2.17	1.8-2.2	1.97	0.003-0.020	0.0133
IV	Thiamine-free	2	2	4.7-7.1	5.57	4.3-6.1	5.12	0.013-0.022	0.0178
				2.1-2.3	2.20	1.8-2.1	1.95	0.016-0.019	0.0175

The data on the excretion of urine following the administration of test doses of *dl*-phenylalanine are given in Table II. It is again evident that there was a greater excretion of hydroxyphenyl compounds by the control rats than by the deficient animals. These differences were statistically significant. The ferric chloride test was positive in only one urine collected after feeding 100 mg. of *dl*-phenylalanine; this was in a deficient animal.

Two possible explanations of these results are apparent, (1) they may reflect the difference in the ages of the animals at the various periods or

(2) they may be due to variations in food intake during the two periods of feeding. This latter possibility is of considerable importance because of the high content of aromatic amino acids in casein.

In a second experiment twelve 30 to 50 gm. rats of the same strain were matched as to sex and divided into two equal groups. These were pair-fed. 24 hour urine specimens were again collected and analyzed before and after test doses of 200 mg. of *dl*-phenylalanine. The data are summarized in Table III. Statistical analysis of these results fails to reveal any significant differences between the animals on the two diets. It was concluded that any small differences which had been observed with the first group of animals had been due to variations in food intake and in the age of the animals at different stages of the experiment. The qualitative test with ferrie chloride was negative in all urines except those

TABLE III

*Analyses of 24 Hour Urine Specimens of Rats Pair-Fed on Diets with and without Thiamine*

Diet	No. of animals	No. of urine speci- mens	Hydroxyphenyl compounds as tyrosine				"Phosphorus equiva- lent"	
			Before ether extraction		After ether extraction			
			Range	Mean	Range	Mean	Range	Mean
			mg.	mg.	mg.	mg.	mg.	mg.
Control	6	6	1.3-3.0	2.85	1.0-3.5	2.75	0.012-0.026	0.0205
" + 200 mg. phenyl- alanine	4	4	3.5-5.5	4.20	2.4-5.1	3.55	0.032-0.081	0.0563
Thiamine-free	6	6	1.0-3.5	2.42	0.9-3.0	2.25	0.006-0.031	0.0182
" + 200 mg. phenylalanine	3	3	3.0-6.2	4.67	2.0-4.7	3.43	0.029-0.109	0.0670

following the administration of additional amounts of *dl*-phenylalanine. In the latter case an intense green was obtained with the urines from both the deficient animals and the pair-fed controls.

#### DISCUSSION

An examination of the data of Closs and Fölling (3) reveals that the differences which they observed were based upon qualitative tests applied to urines of different concentrations and that the animals were not pair-fed. With the feeding régimes and the methods outlined here no evidence was obtained that thiamine-deficient rats were less able to metabolize *dl*-phenylalanine in either the amounts present in a diet containing 25 per cent casein or when additional single doses of 100 to 200 mg. of the amino acid were administered by stomach tube. Thiamine deficiency

in the rat did not, therefore, lead to the appearance in the urine of an increased amount of abnormal metabolic products of the aromatic amino acids. These findings are in agreement with the experiences of Levine *et al.* (9) that supplementary vitamin B complex failed to reduce the excretion of products of tyrosine metabolism by premature infants. It does not appear that thiamine deficiency results in the development of a defect, which can be demonstrated *in vivo*, in the metabolism of phenylalanine in the rat.

#### SUMMARY

In young albino rats maintained on thiamine-free diets there was detected no increased excretion of abnormal metabolic products of tyrosine or phenylalanine. Following the administration by stomach tube of *dl*-phenylalanine to pair-fed deficient and control rats the observed increases in the output of all metabolites tested for were comparable.

The authors wish to express their appreciation to Dr. Albert Segaloff for the gift of the pure strain rats employed in this study and to acknowledge the technical assistance of Miss Caroline Ashley and Mrs. Naomi Dziewiatkowski and the valuable advice of Dr. Paul Densen in the statistical analyses of the results.

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## 2,3-DIPHENACYL AND PARA-SUBSTITUTED DIPHENACYL ASCORBIC ACID\*

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In connection with a study of certain ascorbic acid derivatives in which the enediol system has been stabilized by substitution, we have investigated the reaction of phenacyl halides with ascorbic acid. Phenacyl halides react under rather mild conditions with compounds bearing acidic hydrogen atoms. They have been shown by Reid and coworkers (1-4) and by others (5-9) to yield crystalline derivatives of organic acids in numerous cases.

Ascorbic acid under conditions which do not lead to opening of the lactone ring has 2 acidic hydrogen atoms,  $pK_1 = 4.21$  (10) and  $pK_2 = 11.57$  (11). Hence 1 or possibly 2 moles of reagent halide might be involved in this type of reaction. In this paper we shall present evidence to show that 2,3-disubstituted ascorbic acids have been prepared.

When ascorbic acid was treated with phenacyl bromide, *p*-bromophenacyl bromide, or *p*-phenylphenacyl bromide under the conditions to be described, reaction occurred and crystalline derivatives were obtained in each case. The initial yields were low, but some improvement in yields was effected by carrying out the reaction in the absence of air and by neutralizing the ascorbic acid solutions at 0° with sodium bicarbonate.

The phenacyl derivatives were practically insoluble in water, ether, dilute acid, or alkali, but were appreciably soluble in acetone, dioxane, pyridine, and hot ethanol. The *p*-bromo compound was less soluble than the phenacyl, while the *p*-phenyl derivative was very much less soluble in all reagents.

In order to characterize the compounds a number of preliminary qualitative tests were carried out. All phenacyl derivatives failed to reduce 2,6-dichlorophenol indophenol, which indicates blocking of the enediol system. There was no color with ferric chloride, indicating the absence

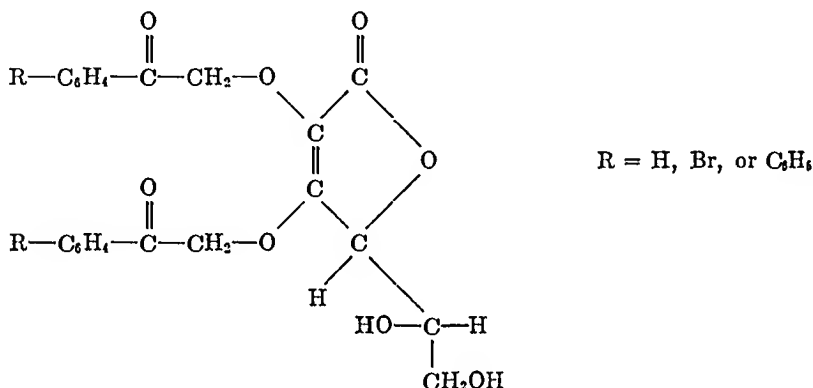
\*From a thesis submitted by Mildred C. Rebstock in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate School of the University of Illinois.

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of enolic hydroxyl groups. No bromine was present in the compound prepared from phenacyl bromide and ascorbic acid. Positive Baeyer's and Fehling's tests were noted, and a reaction with 2,4-dinitrophenylhydrazine, leading to an extremely insoluble derivative, was observed.

To establish the structure of the phenacyl compounds we resorted to analysis and to the preparation and analysis of dibenzoates, diacetates, and isopropylidene derivatives. In addition, Rast molecular weight determinations and the ultraviolet absorption characteristics are in support of the accompanying structure for these new derivatives of ascorbic acid.



While it was extremely unlikely that these compounds would show any antiscorbutic activity, 2,3-diphenacyl ascorbic acid was tested in both curative and preventive experiments with young female guinea pigs. The material failed to show any vitamin properties, but was non-toxic even when fed in amounts as large as 60 mg. per day.

#### EXPERIMENTAL

*2,3-Diphenacyl l-Ascorbic Acid*—The following conditions led to the best yield. The reaction was carried out in a 200 ml. 3-necked flask provided with a reflux condenser and a gas inlet tube leading to the bottom of the flask. 5 gm. (0.028 mole) of *l*-ascorbic acid (Merck, U. S. P.) was added to 25 ml. of ice-cold distilled water which had been deaerated with wet, oxygen-free nitrogen for 15 minutes. Nitrogen was bubbled into the system throughout the reaction. The pH was adjusted to 6.8 (brom-thymol blue) with cold 15 per cent sodium bicarbonate. 10 gm. (0.05 mole) of phenacyl bromide (Eastman) and 50 ml. of 95 per cent ethanol were then added, and the temperature raised quickly to 140° by means of an oil bath. The temperature was allowed to fall to 120° and maintained at 120–125° for 2 hours. At the end of this time the slightly colored reaction mixture was cooled and placed in the refrigerator.

The next day the crystalline material was collected and recrystallized from absolute ethanol. The derivative was obtained in the form of fine white needles. The yield after one recrystallization was 42.7 per cent. An analytical sample which was recrystallized twelve times melted at 136–136.5°.<sup>1</sup>

$C_{22}H_{20}O_8$  (412.36). Calculated, C 64.06, H 4.89; found, C 64.09, H 4.93

*2,3-Di-p-bromophenacyl l-Ascorbic Acid*—The conditions outlined above were adhered to. Recrystallization of this derivative was very difficult, and a good analytical sample was not obtained. Ethanol or methanol was the most satisfactory solvent. In one preparation a yield of 18.4 per cent of twice recrystallized material was obtained. After several recrystallizations the compound melted at 170.5–171°.

$C_{22}H_{18}O_8Br_2$  (570.18). Calculated. C 46.32, H 3.18, Br 23.02  
Found. " 45.59, " 3.37, " 28.57

*2,3-Di-p-phenylphenacyl l-Ascorbic Acid*—A procedure similar to those just described was followed. In this case a precipitate formed on heating to 140° and did not dissolve subsequently. The crude product from 3 gm. of ascorbic acid weighed 4.67 gm. (48.5 per cent yield). It was thoroughly extracted with cold acetone and then recrystallized from hot acetone. After three recrystallizations the material melted at 217.8–219.5°.

$C_{34}H_{28}O_8$  (564.52). Calculated, C 72.32, H 5.00; found, C 72.37, H 5.21

*Attempted Preparation of Monophenacyl l-Ascorbic Acid*—An attempt was made to prepare a mono derivative by carrying out the reaction in the presence of a large excess (5 moles) of ascorbic acid. However, only the disubstitution product was isolated.

From a consideration of the difference in acid strengths of the 2 acidic hydrogen atoms of ascorbic acid one might predict that a mono-substituted derivative could be obtained. In the absence of such a product it may be suggested that the 1st mole of phenacyl halide reacts at carbon atom 3 to form a temporary intermediate. It is possible that the substituent at carbon atom 3 serves to increase the dissociation of the hydrogen at carbon atom 2, with the result that a 2nd mole of phenacyl halide is introduced.

*Properties of Phenacyl and Para-Substituted Phenacyl Derivatives*—The solubility behavior has already been mentioned. The extreme insolubility of the *p*-phenyl compound deserves additional comment. It failed to dissolve in hot ethanol and dissolved slowly in hot acetone, being recrystallized with difficulty from the latter solvent.

<sup>1</sup> All melting points are corrected.

Rast molecular weight determinations (12) were attempted before we succeeded in preparing crystalline derivatives. For 2,3-diphenacyl ascorbic acid the value found was 400 (calculated 412); for 2,3-di-*p*-bromophenacyl ascorbic acid 551 (calculated 570).

The results of certain preliminary qualitative tests have been discussed earlier in this report.

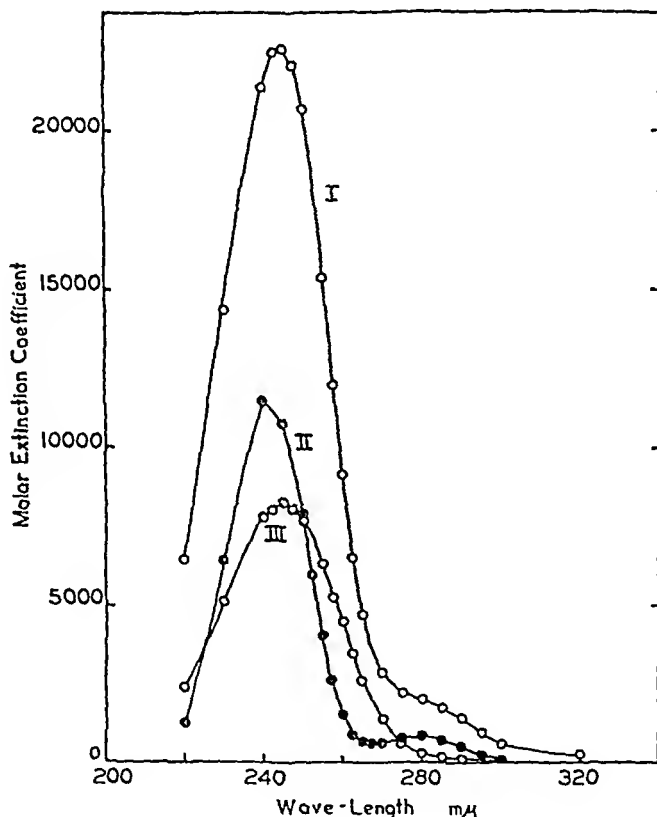


FIG. 1. Ultraviolet absorption curves. Curve I, 2,3-diphenacyl *l*-ascorbic acid,  $6.317 \times 10^{-5}$  M; solvent cell, 1.001 cm.; unknown cell, 1.002 cm. Curve II, acetophenone,  $9.114 \times 10^{-5}$  M, solvent cell, 1.006 cm.; unknown cell, 1.007 cm. Curve III, *l*-ascorbic acid,  $1.056 \times 10^{-5}$  M, solvent cell, 1.001 cm.; unknown cell, 1.007 cm.

The ultraviolet absorption of 2,3-diphenacyl ascorbic acid was studied with the Beckman quartz spectrophotometer. The absorption characteristics are decidedly similar to those of ascorbic acid and certain other ascorbic acid derivatives in which the enediol-lactone system is considered to be intact (see Fig. 1).

In these experiments analytically pure 2,3-diphenacyl ascorbic acid,

redistilled acetophenone, and ascorbic acid (Merck, U. S. P.) were each freshly dissolved in twice distilled 95 per cent ethanol. It can be seen that the absorption maximum for both ascorbic acid and the substituted ascorbic acid is at 245  $m\mu$ . For 2,3-diphenacyl ascorbic acid  $\epsilon_{\text{max.}} = 22,600$ ; for ascorbic acid 8300 (see (13)); for acetophenone  $\epsilon_{240\text{ }m\mu} = 11,480$  (see (14)).

The characteristics of the ultraviolet absorption curves suggest that the essential structure of ascorbic acid is not disturbed in the preparation of the phenacyl derivatives.

*Derivatives of 2,3-Diphenacyl and 2,3-Di-p-bromophenacyl l-Ascorbic Acid*—The 5,6-diacetate of phenacyl ascorbic acid was prepared as follows: 4 ml. of pyridine and 1 ml. of acetic anhydride were cooled to 0°. 300 mg. of phenacyl ascorbic acid were then added. After 30 hours at 0° the reaction mixture was poured into ice-water. Crystallization occurred immediately. The material was recrystallized from methanol four times. It melted at 101–102°.

$C_{24}H_{22}O_{10}$  (496.39). Calculated, C 62.89, H 4.87; found, C 62.75, H 5.02

The acetylation of the *p*-bromo compound was carried out as described above. After several recrystallizations from absolute ethanol the compound melted at 154–154.5°.

$C_{24}H_{22}O_{10}Br_2$  (654.22). Calculated. C 47.73, H 3.39, Br 24.43  
Found. " 47.81, " 3.80, " 23.71

The 5,6-dibenzoates were prepared in anhydrous pyridine with excess benzoyl chloride and kept at 0° for 2 days. In the case of both reactions the systems were poured over ice and, when crystallization failed, extracted with ether. The ether solutions were washed with dilute sulfuric acid, sodium bicarbonate, and water, and then dried over anhydrous sodium sulfate. On removal of the ether crystallization occurred. Both benzoates were recrystallized several times from absolute ethanol and were obtained in the form of fine needles.

The compound, 2,3-diphenacyl-5,6-dibenzoyl ascorbic acid, melted at 119.2–120.2°.

$C_{32}H_{24}O_{10}$  (620.52). Calculated, C 69.68, H 4.55; found, C 69.56, H 4.63

The compound, 2,3-di-*p*-bromophenacyl-5,6-dibenzoyl ascorbic acid, melted at 150–150.5°.

$C_{32}H_{24}O_{10}Br_2$  (778.34). Calculated. C 55.54, H 3.37, Br 20.53  
Found. " 55.62, " 3.60, " 21.29

The preparation of 5,6-isopropylidene derivatives of both the phenacyl and the *p*-bromophenacyl ascorbic acids constitutes perhaps the best evidence for the proposed structure of the phenacyl derivatives of ascorbic acid. These compounds were prepared according to von Vargha (15). Crystallization was induced in each case by the careful addition of low petroleum ether to the acetone solution which had been filtered to remove the copper sulfate and concentrated *in vacuo*. Recrystallization was carried out four times with absolute ethanol in each case.

2,3-Diphenacyl-5,6-isopropylidene ascorbic acid melted at 135.5–136°. It is to be noted that this melting point is only 0.5° lower than that of the starting material. However, a marked depression in a mixed melting point determination indicated that a new compound had been obtained.

$C_{26}H_{24}O_8$  (452.38). Calculated, C 68.36, H 5.35; found, C 66.01, H 5.58

2,3-Di-*p*-bromophenacyl-5,6-isopropylidene ascorbic acid melted at 159.1–159.7°.

$C_{28}H_{22}O_8Br_2$  (610.21). Calculated. C 49.20, H 3.63, Br 26.19  
Found. " 49.64, " 3.94, " 26.08

*Antiscorbutic Properties of 2,3-Diphenacyl l-Ascorbic Acid*—In both curative and preventive experiments on young female guinea pigs, 2,3-diphenacyl ascorbic acid failed to show any antiscorbutic properties. All animals except ascorbic acid controls either died of scurvy or were sacrificed in the last stages of scurvy for blood ascorbic acid analyses by the method of Roe and Kuether (16). Daily doses as large as 60 mg. fed by pipette as a suspension in sucrose solution were ineffective in preventing or alleviating symptoms of scurvy, and appeared to be completely non-toxic. Blood ascorbic acid values in the test animals were in the extreme scurvy range, 0.02 to 0.09 mg. per cent as compared to control values of 0.19 to 0.31 mg. per cent. Thus 2,3-diphenacyl ascorbic acid is unavailable to the guinea pig when administered orally.

#### SUMMARY

The preparation and properties of 2,3-diphenacyl, 2,3-di-*p*-bromophenacyl, and 2,3-di-*p*-phenylphenacyl ascorbic acid have been described.

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## PHOSPHORUS COMPOUNDS IN ANIMAL TISSUES

### I. EXTRACTION AND ESTIMATION OF DESOXYPENTOSE NUCLEIC ACID AND OF PENTOSE NUCLEIC ACID\*

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The phosphorus compounds of animal tissues can be divided into the following four classes on the basis of their analytical properties, acid-soluble, lipid, nucleic acid, and protein. The separation of the first two groups of compounds from the tissue and from each other is well known and consists simply in extracting the tissue with acid to obtain the acid-soluble compounds and in extracting the tissue residue with fat solvents to obtain the lipids.

It is not sufficient merely to separate the phosphorus compounds into these classes. Methods must also be devised for each compound present in each of the four classes of phosphorus compounds. An elaborate analytical scheme has already been proposed for the estimation of fourteen phosphorus compounds found in the acid extracts of animal tissues (20). Preliminary steps towards the determination of the fatty acids in the lipid fraction have been undertaken (12).

The purpose of the present report is to describe (1) a method for the quantitative extraction of the nucleic acids from animal tissues, (2) the integration of this extraction procedure with previous methods for acid-soluble and lipid phosphorus so that all four groups of phosphorus compounds can be determined on a single tissue sample, and (3) the application of colorimetric methods to the estimation of the nucleic acids in the tissue extracts and the correlation of the results of the measurements with the phosphorus found in the extract. The new extraction method consists simply in heating the tissue with 5 per cent trichloroacetic acid after removal of phospholipids and acid-soluble phosphorus compounds.

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Some of the material reported in this paper was taken from a thesis submitted to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

In this paper, "per cent" solutions refer to solutions in which the concentrations are expressed as gm. of material per 100 ml. of solution.

## EXPERIMENTAL

*Methods and Materials*

All of the analytical methods used in this paper were based upon colorimetric reactions and some of the results are expressed as  $E$  values ( $E = \log I_0/I$  where  $I_0/I$  is the ratio of the intensity of the incident light to that of the transmitted light (17)). Measurements of  $E$  values were made with the Ceneo-Sheard spectrophotometer adapted to the use of selected  $13 \times 100$  mm. Pyrex test-tubes. Since the effective thickness of the Pyrex tubes is not known, the  $E$  values reported are not true extinction coefficients but are a characteristic of the set of tubes employed and will vary from one set of tubes to another, depending upon the diameter of the selected set of tubes.

*Colorimetric Methods*—Total phosphorus and nitrogen were measured as described by LePage and Umbreit (20). Desoxypentose nucleic acid (DNA) was measured by the diphenylamine reaction (11) and by the carbazole reaction (11, 15). The orcinol reaction (23) was used to measure pentose nucleic acid (PNA).

*Nucleic Acid*—Seven samples of DNA, two samples of yeast nucleic acid, desoxyribose, ribose, and the desoxyribosides of DNA were tested in the colorimetric reactions.<sup>1</sup> The DNA samples were prepared from thymus, pancreas, and fish sperm by various methods ((14, 16, 21, 28); also Loring<sup>2</sup>).

*Preparation of Tissues*—The tissues used for analysis were obtained from animals fed on the regular laboratory ration of mixed grains. In the case of the samples of rat liver, from which the phospholipids were not removed, and the mouse lung, the tissues were obtained from animals under nembutal anesthesia (50 mg. per kilo) and were frozen in liquid air immediately. The other tissues were obtained from animals killed by decapitation. The frozen or fresh tissues were homogenized in the apparatus of Potter and Elvehjem (25).

*Results**Color Reactions of Nucleic Acids and Nucleic Acid Derivatives<sup>3</sup>*

The nucleic acids used in this study showed considerable variation in their elementary composition but were found to be suitable as standards

<sup>1</sup>The author gratefully acknowledges the following contributions: adenine and thymine desoxyribosides from Dr. T. G. Brady (5); thymine and guanine, thymine, and cytosine desoxyribosides from the collection of the late Dr. P. A. Levene through the courtesy of Dr. A. E. Mirsky; two samples of sodium thymonucleate from Dr. J. P. Greenstein (14); two samples of yeast nucleic acid from Dr. J. A. Bain (2); one sample of sodium desoxyribonucleate (fish sperm) from Dr. H. S. Loring.

<sup>2</sup>Loring, H. S., unpublished method; personal communication.

<sup>3</sup>Recently Cohen (8) described a new colorimetric method for DNA involving heating the nucleic acid with tryptophane and perchloric acid. The sensitivity of

in the colorimetric reactions provided that the color intensities produced were referred to the phosphorus content of the sample (28).

*Diphenylamine Reaction*—The DNA samples gave an average  $E$  value of 0.0224 per microgram of phosphorus. Yeast nucleic acid did not interfere in the reaction. In confirmation of previous observations (8, 24, 31), 1 mole of DNA was found to produce as intense a color as did 2 moles of desoxyribose, desoxyadenosine, or desoxyguanosine. The reaction of the pyrimidine desoxyribosides was slight and incomplete with the diphenylamine reagent (compare (24)).

*Carbazole Reaction*—Both DNA and PNA react with the carbazole reagent. The average  $E$  values per microgram of phosphorus were 0.152 and 0.0124 respectively. In measuring DNA in tissue extracts with this reaction, a correction must be applied for any PNA which may be present. Desoxyribose and the desoxyribosides were tested with this reaction but no simple relation existed between the color intensities produced by these compounds and that produced by DNA.

*Orcinol Reaction*—PNA and DNA gave  $E$  values per microgram of phosphorus of 0.135 and 0.0166 respectively in the orcinol reaction. The reaction of DNA with this reagent cannot be due to impurities in the DNA samples because all samples of DNA gave the same  $E$  values per microgram of phosphorus (see (1)) and because desoxyribose and the desoxyribosides also reacted with the orcinol reagent (1 mole of DNA produced the same color intensity as did 4 moles of desoxyribose, desoxyadenosine, desoxyguanosine, desoxycytidine, or thymidine). In measuring PNA in tissue extracts, a correction must therefore be applied for the reaction of

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this reaction is enormously increased if the fluorescence of the heated solutions is measured (Coleman electronic photofluorometer No. 12, Filters B-1 and PC-1). Straight line relationships were found to exist between the concentration of DNA (as low as 0.5 to 2.5  $\gamma$ ) and the intensity of fluorescence. The reaction was carried out as follows: 0.8 ml. of 70 per cent perchloric acid (Eimer and Amend) were mixed with 0.2 ml. of 1 per cent *l*-tryptophane (Hoffmann-La Roche) and 0.6 ml. of water or DNA solution and the mixture was heated for 20 minutes in a boiling water bath. After addition of 6.4 ml. of water, the intensity of the fluorescence was measured. The intensity of fluorescence of both blanks and of DNA solutions is markedly influenced by the time of heating and the perchloric acid concentration. Adenine and thymine desoxyribosides also yield fluorescent materials when heated with tryptophane and perchloric acid. Ribose nucleic acid does not interfere in the reaction. The fluorescent solutions can be extracted with isoamyl alcohol to yield extracts which give linear relationships between fluorescence intensity and DNA concentration. The fluorescence is characterized by a marked sensitivity to ultraviolet light and the fluorescence intensity decreases considerably upon prolonged exposure to ultraviolet light. Although this reaction was not used for any of the analyses herein reported, it seems possible that the method could be developed into an exceedingly useful and sensitive method for DNA if the steadiness of the galvanometer readings could be increased. This might be accomplished by the use of proper organic solvents to extract the fluorescent material.

any DNA present. 1 mole of PNA produced a color as intense as that produced by 2.6 moles of *d*-ribose.

### *Separation of Nuclcic Acids from Nucleoproteins*

In order to measure nucleic acids in animal tissues colorimetrically, it is necessary to be able to remove the nucleic acids quantitatively from other tissue components. The following experiments show that heating the tissue with 5 per cent trichloroacetic acid at 90° for 15 minutes serves this purpose.

*Splitting of Thymus Nucleohistone by Trichloroacetic Acid*—A thymonucleohistone solution was prepared from rat thymus by the method of

TABLE I

*Splitting of Thymus Nucleohistone (TNH) and of Rat Liver Proteins by Trichloroacetic Acid (TCA)*

Experiment No.	Description	DNA	PNA	P found*	P calculated*
		$\gamma$	$\gamma$	$\gamma$	$\gamma$
1	Original TNH	107		11.2	10.6
	Supernatant from TNH after 15 min. at 90° with 5% TCA	106		10.1	10.5
2†	Rat protein residue heated 15 min. at 90° with 5% TCA	100	309		
	Rat protein residue heated 10 min. at 100° with 2% NaOH	69	339		

DNA = desoxypentose nucleic acid; PNA = pentose nucleic acid.

\* Calculated from the DNA found, assuming that DNA contains 9.89 per cent phosphorus.

† 1 ml. portions of 20 per cent liver homogenates were extracted twice with cold TCA before being heated with TCA or NaOH.

Carter and Hall (6). Aliquots of this solution were heated with an equal volume of 10 per cent trichloroacetic acid for 15 minutes at 90°. Aliquots of the protein-free filtrate were used for the measurement of DNA and total phosphorus. It was possible in this instance to use aliquots of the original thymonucleohistone solution equivalent to the aliquots of the filtrate in the color tests. The results are reported in Experiment 1, Table I. The following conclusions can be drawn: (1) all of the DNA in the thymonucleohistone is removed by the hot trichloroacetic acid treatment, and (2) all of the phosphorus found in the trichloroacetic acid extract is accounted for by the phosphorus in the DNA found.

Further evidence for the splitting of nucleoproteins by trichloroacetic acid has been presented by two groups of workers. Schramm and Dan-

nenberg (29) have demonstrated that ribose nucleic acid is quantitatively split from tobacco mosaic virus by hot trichloroacetic acid.<sup>4</sup> Kaplan and Greenberg (18) observed splitting of rat liver nucleoproteins by cold trichloroacetic acid after long periods of contact (7 days).

*Splitting of Rat Liver Nucleoproteins by Trichloroacetic Acid*—The preceding experiment with thymonucleohistone indicated that one extraction with hot trichloroacetic acid was sufficient to liberate all of the nucleic acid from the protein. This was tested further by using rat liver homogenates as sources of proteins containing both DNA and PNA. The intensities of the orcinol and diphenylamine reactions were considered to be valid measures of PNA and DNA respectively. In Experiment 2, Table I, the yields of DNA and of PNA from rat liver upon heating with trichloroacetic acid and NaOH are compared. The yield of PNA was about the same with both reagents, while less DNA was obtained with NaOH. To test the completeness of the extraction of the nucleic acids from rat liver by hot trichloroacetic acid, the tissue residue from the hot acid treatment was either repeatedly heated with trichloroacetic acid or heated with NaOH. Neither treatment increased the yield of nucleic acid above that observed with a single extraction with hot trichloroacetic acid.

*Time Required for Splitting of Nucleoproteins*—Fig. 1 shows the effect of the time of heating rat tissues with trichloroacetic acid on the intensity of the orcinol and diphenylamine reactions. The experiment was carried out as follows: Acid-soluble and fat-soluble phosphorus compounds were removed from 1.0 ml. portions of a homogenate containing 194 mg. of rat liver and 32 mg. of rat thymus (see the proposed method of analysis for the detailed procedure). The tissue residues were then heated with 5.0 ml. of 5 per cent trichloroacetic acid for varying periods of time. After being cooled and filtered, aliquots of the extract were used for the diphenylamine and orcinol reactions. The resulting *E* values are plotted in Fig. 1. Maximum amounts of PNA and DNA were obtained with a heating period of 10 minutes or longer.

*Concentration of Trichloroacetic Acid Required for Splitting of Nucleoproteins*—Acid- and fat-soluble phosphorus compounds were removed from portions of a homogenate containing 194 mg. of rat liver and 32 mg. of thymus as in the preceding experiment. The tissue residues were then heated for 15 minutes at 90° with 5.0 ml. of trichloroacetic acid solutions of varying concentration. After the heating period, 100 per cent trichloroacetic acid was added so that the final concentration of trichloroacetic acid in each mixture was 10 per cent. Aliquots of the filtrates were used in the diphenylamine and orcinol reactions. The results are plotted in Fig. 2

<sup>4</sup> All of the data reported in the present paper were obtained without any knowledge of the report of Schramm and Dannenberg (29), which was not concerned with the analytical possibilities of the findings.

and show that a trichloroacetic acid concentration of 3 to 4 per cent is sufficient for maximum yields of nucleic acids. The acidity is the important factor in the splitting of nucleoproteins; if neutralized trichloroacetic acid is used, no splitting occurs. The nucleic acids liberated at zero trichloroacetic acid concentration (Fig. 2) can perhaps be attributed to traces of trichloroacetic acid remaining from the preliminary washing

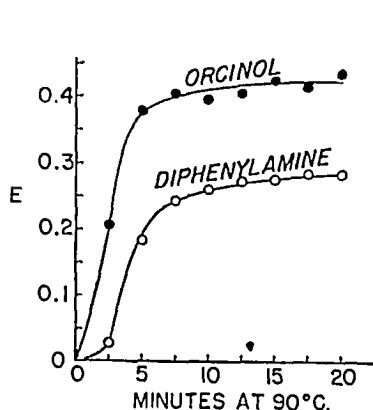


FIG. 1

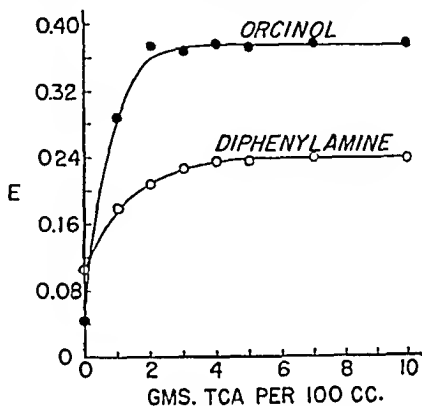


FIG. 2

FIG. 1. The effect of the time of heating rat tissues with 5 per cent trichloroacetic acid on the intensities of the orcinol and the diphenylamine reactions. See the text for detailed description.

FIG. 2. The effect of heating rat tissues with various concentrations of trichloroacetic acid on the intensities of the orcinol and diphenylamine reactions. See the text for detailed description.

of the tissue residue with cold trichloroacetic acid after lipid removal (see the proposed method).

### *Proposed Method of Analysis*

For purposes of convenience, the analytical method will be described for rat liver. All steps are carried out in 16 mm. Pyrex test-tubes cut to a length of 100 mm. and provided with a pour out lip. Homogenization of the tissue (25) is especially important because it provides an extremely fine tissue suspension which facilitates extraction.

*I. Removal of Acid-Soluble Phosphorus Compounds*—1 ml. of a 20 per cent rat liver homogenate is mixed with 2.5 ml. of cold 10 per cent trichloroacetic acid and centrifuged. The precipitate is resuspended in 2.5 ml. of cold 10 per cent trichloroacetic acid and centrifuged. The trichloroacetic acid extracts are combined to form the acid-soluble phosphorus fraction. The compounds in this extract can be fractionated as described by LePage and Umbreit (20).

*II. Removal of Phospholipids*—The tissue residue from (I) is suspended in 1.0 ml. of water, mixed with 4.0 ml. of 95 per cent ethyl alcohol, and centrifuged. The residue is resuspended in 5.0 ml. of alcohol and centrifuged. These steps are used to remove traces of trichloroacetic acid left in the tissue residue from (I). The tissue residue is now boiled three times for 3 minutes each with 5.0 ml. portions of 3:1 alcohol-ether (4). The addition of a few small pieces of pumice-stone greatly facilitates boiling. The alcohol and the alcohol-ether extracts are combined to form the phospholipid fraction.

*III. Removal of Nucleic Acids*—The tissue residue from (II) is suspended in 1.2 ml. of water, mixed with 1.3 ml. of cold 10 per cent trichloroacetic acid, and centrifuged. The residue is suspended in 5.0 ml. of 5 per cent trichloroacetic acid, heated 15 minutes at 90°, cooled, and centrifuged. The residue is resuspended in 2.5 ml. of 5 per cent trichloroacetic acid and centrifuged. The trichloroacetic acid extracts are combined to form the nucleic acid extract.<sup>5</sup>

*IV. Phosphoprotein*—The residue from (III) is mixed with 5.0 ml. of 2 per cent NaOH and dissolved by heating in a boiling water bath for 10 minutes. This fraction is considered to be the phosphoprotein fraction.

*Aliquots Used for Analysis*—0.5 ml. aliquots of each extract were used for total phosphorus determinations. The following aliquots of extract (III) were used for nucleic acid measurements: diphenylamine reaction, 1.0 ml.; carbazole and orcinol reactions, 0.2 ml. each.

*Calculation of Nucleic Acid Results*—Since two of the nucleic acid analyses involve corrections, the calculations will be outlined briefly (see the "Results" for the color reaction constants).

$$\begin{aligned}
 A &= \text{micrograms DNA P per ml. solution} = \frac{E_{\text{diphenylamine}}}{0.0224} \\
 B &= \quad \quad \text{PNA " " 0.2 ml.} = \frac{E_{\text{orcinol}} - (0.2A \times 0.0166)}{0.135} \\
 C &= \quad \quad \text{DNA " " 0.2 " } = \frac{E_{\text{carbazole}} - 0.0124B}{0.152}
 \end{aligned}$$

The results can be converted to micrograms of DNA or PNA by dividing by 0.099 and 0.095 respectively (15).

<sup>5</sup>The sensitivity and the accuracy of the nucleic acid analyses would be greatly increased if a means of precipitating the nucleic acids quantitatively from the trichloroacetic acid extract could be devised. Freshly precipitated  $\text{Zn}(\text{OH})_2$  was found effective in precipitating DNA (prepared by the method of Hammarsten (16)) in concentrations as low as 20  $\gamma$  per ml. The  $\text{Zn}(\text{OH})_2$  precipitation method failed to precipitate the nucleic acids quantitatively from hot trichloroacetic acid extracts of rat liver, however (28).



*Application of Analytical Method*

The results of the analysis of rat liver, mouse lung, and rat brain are summarized in Table II. In some cases, the alcohol and the alcohol-ether extractions were omitted.

Several points should be emphasized in connection with Table II. In the first place, extraction of the phospholipids prior to the nucleic acids

TABLE II  
*Analysis of Animal Tissues for Nucleic Acids by Means of Hot Trichloroacetic Acid Method*

Tissue	No. of analyses	Mg. per 100 gm. wet tissue						
		PNA	DNA (diphenylamine reaction)	DNA (carbazole reaction)	P found	P calculated*	N found	N calculated*
Rat liver†	7	754	236	254	105	95	191	161
		657-	196-	240-	92-	85-	173-	145-
		910	264	292	127	106	234	179
Rat liver	4	834	213		98	100		
		668-	198-		83-	85-		
		1050	221		115	118		
Mouse lung†	8	238	586	591	68	81	208	137
		194-	399-	527-	38-	56-	143-	94-
		330	879	650	100	118	283	201
Rat brain†	3	195	150		71.6	34.3	100	58
		183-	138-		69.0-	31.9-	98-	54-
		204	174		74.0	36.3	103	61
Rat brain	3	184	124		29.5	29.7	83	53
		155-	120-		28.0-	27.0-	81-	50-
		208	130		31.5	32.7	85	55

DNA = desoxypentose nucleic acid; PNA = pentose nucleic acid.

The average values are given in bold-faced type.

\* These calculations were made on the assumption that DNA contained 9.89 per cent phosphorus and 16.76 per cent nitrogen and that PNA contained 9.5 per cent phosphorus and 16.1 per cent nitrogen.

† In these analyses the phospholipids were not extracted prior to the nucleic acid extraction.

had little effect on the amount of nucleic acid revealed by the colorimetric PNA or DNA determinations. The higher content of PNA in the lipid-extracted rat liver samples may be due to the smaller number of analyses or to the fact that the analyses were not made on the same samples of tissue. The brain analyses with and without lipid removal were made on the same samples of tissue and here the colorimetrically determined nucleic acids are in excellent agreement. The extraction of the lipids did make a difference in the amount of phosphorus in the trichloroacetic acid extracts accounted for by nucleic acids. The difference was small in

the case of rat liver: 92 per cent of the phosphorus was accounted for in the unextracted tissue, while 102 per cent was accounted for in the lipid-extracted livers. The difference was striking in the case of rat brain: 101 per cent of the phosphorus was accounted for by nucleic acids in the lipid-extracted brains, while only 48 per cent was accounted for in the unextracted tissue.

Although all of the phosphorus in the trichloroacetic acid extracts can be accounted for by nucleic acids, this is not the case for nitrogen. The nitrogen found was always higher than that calculated from the nucleic acids found (Table II). This may mean either that the nucleic acids in these tissues do not conform to the accepted tetranucleotide structure of a nucleic acid or that some other nitrogen compound is extracted by the hot acid.

DNA has been measured by two independent methods, the diphenylamine and the carbazole reactions, and the results check very well (see Table II). This finding indicates that the sugars in the DNA split from the protein by the trichloroacetic acid are apparently intact, because Dische has demonstrated that the diphenylamine reaction involves only the purine-bound sugars of DNA, while the carbazole reaction involves only the pyrimidine-bound sugars (11).

Further evidence for the effectiveness of the new method is provided by the fact that the DNA and the nucleic acid phosphorus found in the hot trichloroacetic acid extracts agree well with the values obtained by other workers using different methods (3, 9, 10, 13, 19, 22, 26, 27).

#### DISCUSSION

A method has been described for the extraction and measurement of nucleic acids in animal tissues. A variety of evidence has been presented for the quantitative nature of the extraction. The new method furnishes data not only on nucleic acids, but also data on acid-soluble, lipid, and protein phosphorus. This feature is of especial significance in work with radioactive phosphorus, because in this case it is desirable to be able to separate the four classes of phosphorus compounds in a single tissue sample.

No attempt was made to study the splitting of nucleoproteins by other acids, since it was felt that few if any acids could be found which had all of the desirable properties of trichloroacetic acid. Other acids such as hydrochloric (9, 30) and malonic (7) have been used to split nucleoproteins, but these acids are much poorer protein precipitants than is trichloroacetic acid.

The author is indebted to Dr. V. R. Potter for the suggestion of this problem and for his continued interest in this work.

## SUMMARY

1. Several nucleic acid samples, desoxyribose, ribose, and several deoxyribosides were tested and compared in their reactions with the diphenylamine, orcinol, and carbazole reagents.

2. It was found that a single extraction with hot trichloroacetic acid would quantitatively remove nucleic acids from thymonucleohistone and from animal tissues.

3. The effect of the trichloroacetic acid concentration and of the time of heating with trichloroacetic acid on the amounts of nucleic acids extracted from liver tissues was studied.

4. The hot trichloroacetic acid extraction was combined with known methods to produce a scheme for the separation of the phosphorus compounds of animal tissues into four groups: acid-soluble, lipid, nucleic acid, and protein.

5. Rat liver, rat brain, and mouse lung were analyzed for nucleic acids.

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# THE VITAMIN A ACTIVITY OF NEO- $\beta$ -CAROTENE U AND ITS STERIC REARRANGEMENT IN THE DIGESTIVE TRACT OF RATS

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Recently Polgár and Zechmeister (1) pointed out that all *trans*- $\beta$ -carotene can exist in a number of other stereoisomeric forms. Kemmerer and Fraps (2, 3) have shown that carotene extracts of plants contain two of these stereoisomers, neo- $\beta$ -carotene B and neo- $\beta$ -carotene U, in appreciable quantities. These authors reported (2) that neo- $\beta$ -carotene B had one-half the vitamin A activity of all *trans*- $\beta$ -carotene and that neo- $\beta$ -carotene U did not possess vitamin A activity. Deuel, Johnston, Sumner, Polgár, and Zechmeister (4) have since reported that neo- $\beta$ -carotene U possesses 38 per cent of the vitamin A activity of all *trans*- $\beta$ -carotene. Because the results of these authors did not agree with our findings and because neo- $\beta$ -carotene U is present in appreciable quantities in many plant materials, we have reinvestigated the biological activity of neo- $\beta$ -carotene U and this paper reports the findings.

## EXPERIMENTAL

### *Biological Activity*

Two sources of neo- $\beta$ -carotene U were used. One was prepared by iodine catalysis of crystalline carotene (1) and the other was extracted from dehydrated alfalfa leaf meal (2). The neo- $\beta$ -carotene U was separated and purified by chromatographing on a column of calcium hydroxide (2). Both preparations were finally dissolved in Wesson oil and were kept in the refrigerator during the biological assay. The quantity of neo- $\beta$ -carotene U in the Wesson oil solutions was determined by dissolving 1 gm. of the oil solution in 100 ml. of hexane and reading the densities at 445 and 475  $m\mu$  with a Beckman and a Bausch and Lomb spectrophotometer. The micrograms per gm. of neo- $\beta$ -carotene U were calculated from the densities by use of molecular extinction coefficients taken from absorption curves published by Zechmeister and Polgár (5).

The purity of the neo- $\beta$ -carotene U was checked by chromatographic and spectrophotometric analyses. The absorption maxima in hexane were 474 and 446  $m\mu$  as compared with 474 and 446 to 447  $m\mu$  reported by Zechmeister and Polgár (5). The solutions were examined spectrophotometri-

cally and chromatographically again at the end of the biological assay. The amount of neo- $\beta$ -carotene U in the solutions had not been reduced, and stereoisomerization had not taken place.

The U. S. P. method, slightly modified (6), was used for determining the vitamin A potency of the neo- $\beta$ -carotene U. Crystalline carotene in Wesson oil was used as a standard and had the following percentage composition: impurity A 1.6,  $\beta$ -carotene 82.6, neo- $\beta$ -carotene B 1.6, and  $\alpha$ -carotene 14.2. According to Deuel, Sumner, Johnston, Polgár, and Zechmeister (7),  $\alpha$ -carotene has 53 per cent of the vitamin A potency of  $\beta$ -carotene and, according to Kemmerer and Fraps, neo- $\beta$ -carotene B has one-half the potency of  $\beta$ -carotene. The  $\beta$ -carotene equivalent of the carotene standard, then, is the  $\beta$ -carotene plus one-half the  $\alpha$ -carotene plus one-half the neo- $\beta$ -carotene B, or 90.5 per cent of  $\beta$ -carotene. The

TABLE I  
*Vitamin A Activity of Neo- $\beta$ -carotene U*

Supplement	Dose per day	No. of rats at end of test	Average increase in weight in 4 wks.	Vitamin A potency as per cent of $\beta$ -carotene
	$\gamma$		gm.	
$\beta$ -Carotene (equivalent)	0.6	6	23.8	100
	0.8	8	37.0	
	1.0	8	48.6	
Neo- $\beta$ -carotene U (iodine catalysis)	1.6	6	21.2	(35)*
	3.2	10	30.8	22
" " (from alfalfa)	1.6	7	19.6	(34)*
	3.2	10	38.1	25

\* Calculated by extending the limits of the standard curve.

standard solution was freshly prepared every 2 weeks. Both preparations of neo- $\beta$ -carotene U were fed at daily levels of 1.6 and 3.2  $\gamma$ .

In order to calculate the vitamin A potency of the neo- $\beta$ -carotene U, the gains in weights of the rats on the standard carotene were plotted against the log of the dosage. The vitamin A potency of the neo- $\beta$ -carotene U, in units of  $\beta$ -carotene, was interpolated from this curve.

The results of the biological assays are given in Table I. At the 3.2  $\gamma$  level of feeding, the vitamin A potency of neo- $\beta$ -carotene U prepared by iodine catalysis of  $\beta$ -carotene was 22 per cent of that of  $\beta$ -carotene and the vitamin A potency of neo- $\beta$ -carotene U extracted from alfalfa was 25 per cent of  $\beta$ -carotene. At the 1.6  $\gamma$  level of feeding, the growth rates of the rats were below the limits of the standard curve and are therefore not included in the final calculation of vitamin A potency. However,

for the sake of comparison, the limits of the curve were extended theoretically to include these growth rates. The results thus obtained (Table I) are only approximations and are higher than the others.

### *Stereoisomerization in Digestive Tract*

In order to determine whether the vitamin A activity of neo- $\beta$ -carotene U was due to steric rearrangement in the digestive tract, two experiments were carried out. In the first experiment, neo- $\beta$ -carotene U in Wesson oil was fed to rats and the resulting carotenoids in the feces determined. Three rats 28 to 30 days old were placed in individual cages and fed the U. S. P. vitamin A test diet for a preliminary period of 3 days. This technique, according to previous work (8), removes all but perhaps traces of carotene from the digestive tract. After the preliminary period, the rats were continued on the U. S. P. diet and also fed 47  $\gamma$  of neo- $\beta$ -carotene U in Wesson oil, daily, two feeding periods. During the first period the rats were fed the neo- $\beta$ -carotene U solution for 3 days. The feces were collected twice daily, stored at 5°, and chromatographic analyses made by treatment with alcoholic potassium hydroxide at room temperature and separation on calcium hydroxide as previously described (2). During the second period, which was a continuation of the first period, the rats were fed the neo- $\beta$ -carotene U solution for 3 days and then continued on the basal diet for 2 additional days. The feces for this 5 day period were collected and treated and analyzed as in the first feeding period.

In the second experiment, neo- $\beta$ -carotene U in Wesson oil was introduced into the stomach of rats with a catheter, and the carotenoid constituents determined after the material had remained in the digestive tract for short periods of time. Rats 35 days old were fed as in the first experiment to remove carotene from their digestive tracts. They were fasted overnight (15 hours) and then lightly anesthetized with ethyl ether. The catheter was introduced into the stomach by way of the mouth, and 1.3 ml. of the oil solution of neo- $\beta$ -carotene U (120  $\gamma$ ) administered. All the rats used were out of the anesthetic immediately after they had been given the oil solution. The oil solution was administered in less than 1 minute. After 0, 4, or 6 hours had elapsed, the rats were killed and the stomach and intestines and their contents analyzed chromatographic ly for carotenoid constituents (2).

The results of these two experiments are given in Table II. It is apparent that steric rearrangement of neo- $\beta$ -carotene U into carotene takes place in the digestive tract. After the neo- $\beta$ -carotene U solution had remained in the digestive tract for 6 hours, the carotenoids in addition to neo- $\beta$ -carotene U consisted of 17.7 per cent  $\beta$ -carotene, 11.5 per cent neo- $\beta$ -carotene B, and 12.6 per cent impurity A. This is in contrast to



only 3 per cent  $\beta$ -carotene at 0 hour. Also, at 6 hours, twice as many micrograms of  $\beta$ -carotene were recovered as at 0 hour. Even greater steric rearrangement was found in the feces, the crude carotene of which contained 45 per cent  $\beta$ -carotene.

TABLE II  
*Stereoisomerization of Neo- $\beta$ -carotene U in Digestive Tracts of Rats*

		Neo- $\beta$ -carotene U given	Total carotenoid re- covered	$\beta$ Carotene recovered	Constituents in crude carotene extracts			
		$\gamma$	$\gamma$	$\gamma$	Impurity A per cent	Neo- $\beta$ -caro- tene U per cent	$\beta$ -Carotene per cent	Neo $\beta$ -caro- tene B per cent
Neo- $\beta$ -carotene U in Wesson oil	0 hr. in digestive tract	120	107	3.0	4.0	93.0	3.0	
	4 hrs. " " "	120	76	8.0	5.6	73.2	11.3	9.9
	6 " " " "	120	45	7.0	12.6	58.2	17.7	11.5
Feces from 3 rats fed neo- $\beta$ -carotene U	1st feeding period	423	50	22.5	14.5	28.1	44.9	12.5
	2nd " " "	423	88	40.0	10.4	35.8	45.4	8.4

TABLE III  
*Effect of New Value for Vitamin A Activity of Neo- $\beta$ -carotene U upon  $\beta$ -Carotene  
Equivalents Previously Published (9, 10)*

	No. of samples averaged	Neo- $\beta$ - carotene U	$\beta$ -Carotene equivalent	
			As published	Corrected
		per cent	per cent	per cent
Leafy vegetables, raw....	16	10	80	83
" " cooked....	8	13	73	76
" " dehydrated .	4	11	74	77
" " canned....	6	20	56	61
Apricots, canned....	3	2	66	67
Squash and pumpkin, cooked.....	3	5	55	56
Fresh grasses.....	31	12	77	80
Dormant grasses.....	13	16	56	60
Silage.....	8	14	32	36

In order to be certain that the steric changes in these two experiments were not caused by manipulations in the method used, an aliquot of the Wesson oil solution of neo- $\beta$ -carotene U was treated with alcoholic potassium hydroxide and separated on calcium hydroxide in the same manner as were the feces and the digestive tracts. No steric changes were observed.

## DISCUSSION

On the basis of the data obtained, neo- $\beta$ -carotene U has one-fourth the vitamin A potency of  $\beta$ -carotene. This potency is due to part of the neo- $\beta$ -carotene U being transformed into  $\beta$ -carotene in the digestive tract of the rats. The  $\beta$ -carotene equivalent of a plant extract, as accurately as it can be ascertained at the present time, is the  $\beta$ -carotene plus one-half the neo- $\beta$ -carotene B plus one-half the  $\alpha$ -carotene plus one-fourth the neo- $\beta$ -carotene U. The  $\beta$ -carotene equivalents given for carotene extracts of various plants in our previous publications (9, 10) are slightly low (Table III). The greatest differences occur with dormant grasses, cooked leafy vegetables, and silage.

## SUMMARY

Neo- $\beta$ -carotene U prepared by iodine catalyses of  $\beta$ -carotene had for rats 22 per cent of the vitamin A activity of all *trans*- $\beta$ -carotene, and neo- $\beta$ -carotene U extracted from alfalfa had 25 per cent of the activity of all *trans*- $\beta$ -carotene. This activity was due to steric rearrangement of part of the neo- $\beta$ -carotene U into  $\beta$ -carotene and neo- $\beta$ -carotene B in the digestive tract of the rats.

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# FUNCTION OF THE VITAMIN B<sub>6</sub> GROUP: PYRIDOXAL PHOSPHATE (CODECARBOXYLASE) IN TRANSAMINATION

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Vitamin B<sub>6</sub> has for some time been implicated in protein metabolism. More recently a specific function of pyridoxal in the coenzyme of tyrosine decarboxylase has been found (1), and the discovery of this function extended to include amino acid decarboxylases in general (2, 3). All members of the vitamin B<sub>6</sub> group (pyridoxine, pyridoxal,<sup>1</sup> pyridoxamine) are converted into this coenzyme (termed *codecarboxylase* (4) in recognition of its function in amino acid decarboxylases) by those organisms using them as a source of vitamin B<sub>6</sub> (5). The amino acid decarboxylases so far shown to require the coenzyme include tyrosine (2, 5, 6), lysine (7), arginine (2, 3), ornithine (2), glutamic acid (3), and dopa (3, 4-dihydroxy-phenylalanine<sup>2</sup>).

The exact structure of the coenzyme is not certain but the properties are sufficiently well known to indicate that it is a phosphorylated derivative of pyridoxal and to suggest the possible position of linkage. The naturally occurring and the synthetic preparations of coenzyme, which possess similar properties (2, 8), are referred to here as pyridoxal phosphate, or codecarboxylase, without further specification of structure. The function of codecarboxylase in amino acid decarboxylation assures it a place in any consideration of protein metabolism but does not necessarily mean that this is the only function of the vitamin B<sub>6</sub> group.

Schlenk and Snell (9) have recently reported that tissues of rats on a vitamin B<sub>6</sub>-deficient diet possess low transaminase activity as compared with those of animals supplied adequate amounts of vitamin B<sub>6</sub>. The transamination rate of deficient tissues could at times be stimulated by the addition of pyridoxal and adenosine triphosphate, a procedure known to activate the tyrosine decarboxylase system of dried cell preparations of *Streptococcus faecalis* (6).

Snell (10) has also demonstrated interconversion of pyridoxal and pyridoxamine by heating with amino and keto acids respectively, and has

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<sup>1</sup>We are indebted to the Research Laboratories of Merck and Company, Inc., for the pyridoxal used in these experiments.

<sup>2</sup>Green, D. E., personal communication.

suggested that biological reactions to which these systems are analogous may exist. The biological reaction of the conversion of pyridoxamine into pyridoxal (in the form of pyridoxal phosphate) in the presence of pyruvate has been demonstrated by resting cells of *Streptococcus faecalis* (5).

Studies, to date, of transaminase enzymes have left some uncertainty as to the presence of a coenzyme; Kritzmann (11) reported that a coenzyme is required for the aspartic transaminase, whereas Cohen (12) was unable to find evidence that a coenzyme is involved in transamination. Leloir and Green (13) have isolated two transaminase enzymes in highly purified condition and reported that no evidence was found of dissociable prosthetic groups or of significant amounts of B complex vitamins.<sup>3</sup>

The recent demonstration of transamination in lactic acid bacteria (14) offers a new approach to this problem. Cohen and Lichstein (15), using *Streptococcus faecalis* R grown in the pyridoxine-deficient medium (16), were able to demonstrate a decreased rate of tyrosine decarboxylation but not of transamination. They therefore concluded that vitamin B<sub>6</sub> is not involved in transamination. It now appears that their results may be questioned on the basis of the presence of small amounts of pyridoxine in the medium, which, although not enough to saturate the tyrosine enzyme, may have been sufficient for activity of the transamination system.

In this study, vitamin B<sub>6</sub> in the form of pyridoxal phosphate (codecarboxylase) has been shown to function in transamination. Improved methods for the production of an active transaminase system have been found, and dried cell preparations and cell-free transaminase enzymes have been prepared. Further knowledge of the properties of the bacterial transaminase enzymes has been obtained.

### Methods

*Growth of Culture and Preparation of Enzymes*—*Streptococcus faecalis* R (American Type Culture Collection, No. 8043) was used. Cells of this organism which possess very active transaminase enzymes for the systems

- (1) Glutamic acid + oxalacetic acid  $\rightleftharpoons$  aspartic acid +  $\alpha$ -ketoglutaric acid  
(2) Glutamic acid + pyruvic acid  $\rightleftharpoons$  alanine +  $\alpha$ -ketoglutaric acid

can be obtained by growing the culture in a medium composed of 1 per cent each of tryptone, yeast extract, and K<sub>2</sub>HPO<sub>4</sub> and 0.2 per cent glucose. In this medium the final pH is 6.8 to 7.0. The neutral medium yields cells with distinctly more active transamination systems than the medium

<sup>3</sup> Dr. Green has now been able to recover pyridoxal phosphate from the purified transaminase and to assay the coenzyme with dopa decarboxylase apoenzyme (personal communication).

with a final pH of 4.5 to 5, as used for the maximum production of tyrosine decarboxylase (16). The cells were harvested from the neutral medium after 12 to 15 hours incubation at 37° by centrifugation with a Sharples supercentrifuge and washed once with 0.9 per cent saline. Dried cell preparations were obtained by suspending the washed cells in distilled water and drying in shallow layers over Drierite in vacuum desiccators.

The transaminase enzymes were liberated from the cells by the autolysis procedure which Gale and Epps (7) previously used to obtain cell-free amino acid decarboxylases (3, 7, 8). The autolysates could be freed of cell debris by centrifugation with a Beams ultracentrifuge to yield water-clear cell-free enzyme preparations. Resolution of the enzymes was accomplished by aging; this will be discussed with the data.

Transaminase apoenzymes were produced by growing the culture in a synthetic medium modified from that used for the production of tyrosine apoenzyme (17). The changes in the medium were a decrease in the level of glucose to 0.3 per cent and an increase in the buffer content to 1 per cent. The culture was neutralized with sterile  $\text{N NaOH}$  after growth was well under way so that the pH was maintained between 7.5 and 6.8. The apoenzyme was obtained by growing the culture in the medium with alanine instead of vitamin  $\text{B}_6$  and the holoenzyme (intact enzyme) was obtained from cells grown in the same medium with 3  $\gamma$  of pyridoxal per 10 ml.

*Transamination Experiments and Analysis for Amino Acids*—Incubations for transamination were carried out in Y-shaped tubes, the cell suspensions (or cell preparations), buffer, and other additions being placed in one arm and the substrates, as the neutral salts, in the other. For the experiments reported in this paper all incubations were under anaerobic conditions at 37°. With this culture the reactions could as well be run without anaerobic precautions. Several Y-tubes were connected to a manifold, placed in a water bath, evacuated, and filled with nitrogen. After 10 minutes equilibration, the contents of the two arms were mixed, and the transamination reaction was allowed to proceed for the desired time and then stopped by the addition of 0.5 ml. of 10 per cent sulfuric acid.

Aspartic acid was determined by the chloramine-T method of Cohen (12). Glutamic acid was determined by the use of a specific glutamic acid decarboxylase preparation from *Escherichia coli* (3). The oxalacetic acid was removed by heating in acid solution, after which the samples were adjusted to pH 5.0 for analysis.

#### EXPERIMENTAL

Inasmuch as highly active transaminase enzymes have only recently been shown to exist in bacteria (14), and because the systems previously studied were accompanied by an aspartic acid decarboxylase which ren-

dered both the analysis and interpretation of the results difficult, it seemed desirable to extend the studies of bacterial transamination to systems not so complicated by side reactions. Therefore, the transaminase systems of *Streptococcus faecalis* R were studied in living cells, and then extended to dried cell preparations, and to cell-free water-clear preparations in which permeability and related phenomena were eliminated.

A second series of experiments dealt with the function of pyridoxal phosphate (codecarboxylase) as the coenzyme of transamination.

*Transaminase Systems of Streptococcus faecalis*—A study of the formation of aspartic acid, by the method of Cohen (12), with a dried cell preparation of *Streptococcus faecalis*, has shown that quantities of the preparation beyond 20 mg. do not give appreciably greater amounts of aspartic acid after 30 and 60 minutes incubation (Fig. 1). When transamination was

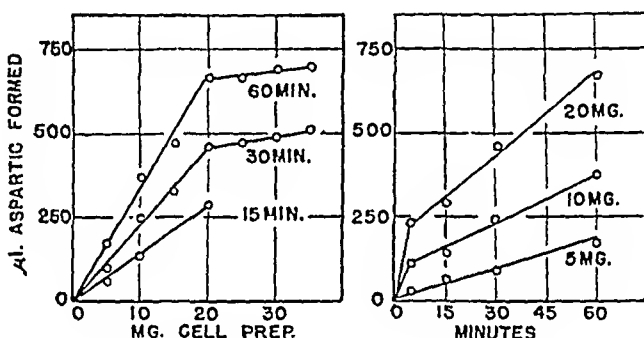


FIG. 1. Transamination by dried cell preparation of *Streptococcus faecalis* R. Dried cell preparation as indicated; 2 ml. of 0.1 M phosphate buffer, pH 8.3, 0.5 ml. of 0.12 M glutamate (1340 microliters), 0.3 ml. of 0.2 M oxalacetate; incubated under  $N_2$  at 37° for the times indicated.

studied for various lengths of time with 10 and 20 mg. of the preparation, measurable amounts of aspartic acid were formed in less than 10 minutes. A decreased rate of reaction occurred with longer periods of incubation, presumably because the reaction approached equilibrium. The rate of the two transamination reactions, (1) glutamate-aspartate and (2) glutamate-alanine, were studied in both the forward and backward directions with the dried cell preparations. The data in Table I show that the preparations catalyzed both reactions with reasonable rates. As had been previously reported for animal tissue (12), the rate of the forward reactions, i.e. glutamate utilization, is greater than the reverse reactions. The rates obtained in the 5 minute incubation period may well be near the maximum values obtainable with these cells, for the reactions had not proceeded far enough to approach equilibrium and thus decrease the reaction rate.

The equilibrium of the glutamic-aspartic reaction is shown in Fig. 2; the forward reaction was measured by the determination of aspartic acid

TABLE I

*Transamination Rates of Dried Cell Preparations of Streptococcus faecalis R*  
- 10 mg. of dried cells, 2 ml. of 0.1 M phosphate buffer, pH 8.3, 0.5 ml. of 0.12 M amino acid, 0.3 ml. of 0.2 M keto acid; incubation at 37° under nitrogen.

Reaction	Incubation time	Glutamic acid formed or used	Transamination	$Q_T (N)^*$
	min.	microliters	per cent	
Glutamate + oxalacetate	5	127	10.2	760
	15	192	15.3	385
	30	310	24.8	310
Aspartate + $\alpha$ -ketoglutarate	30	132	10.6	130
	60	190	14.8	95
	120	266	20.8	65
Glutamate + pyruvate	5	98	7.8	590
	15	150	12.0	300
	30	190	15.3	190
Alanine + $\alpha$ -ketoglutarate	5	11	0.9	65
	15	14	1.0	30
	30	14	1.0	15

\*  $Q_T (N)$  = microliters of substrate transaminated per hour per mg. of cell nitrogen.

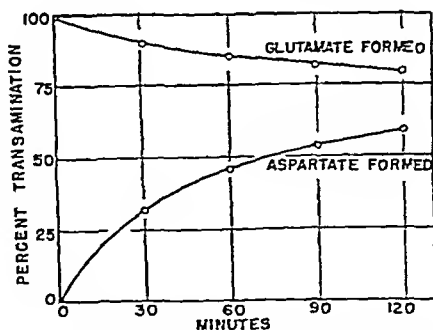


FIG. 2. Reversibility of glutamate-aspartate transaminase of *Streptococcus faecalis R*. 20 mg. of dried cell preparation; 2 ml. of 0.1 M phosphate buffer, pH 8.3. For the lower curve, 0.5 ml. of 0.12 M glutamate, 0.3 ml. of 0.2 M oxalacetate; analyzed by the chloramine-T method. For the upper curve, 0.5 ml. of 0.25 M *D*-aspartate, 0.3 ml. of 0.2 M  $\alpha$ -ketoglutarate; analyzed by the glutamic decarboxylase method. Incubation at 37° under nitrogen.

formation, with glutamic and oxalacetic acids as substrates, and the reverse reaction was measured by the appearance of glutamic acid, with aspartic



and  $\alpha$ -ketoglutaric acids as substrates. In these studies *dl*-aspartate was used in double the desired concentration of the natural isomer and the assumption made that the unnatural form does not interfere with the reaction. The curves are virtually identical with those reported by Albaum and Cohen (18) for oat seedlings. The fact that the two curves do not approach one another more closely may indicate that one or more of the substrates is involved in other processes, the products from which

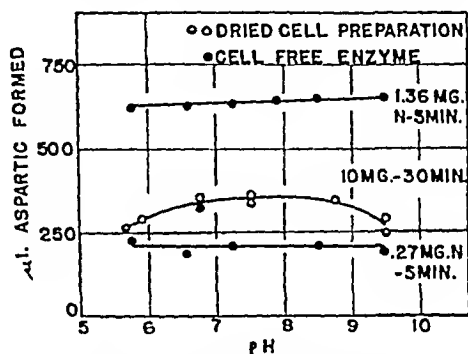


FIG. 3. Influence of pH on glutamate-aspartate transaminase activity. 10 mg. of dried cell preparation, 0.27 or 1.36 mg. of N of cell-free transaminase, 2 ml. of 0.1 M phosphate buffer, pH 8.3, 0.5 ml. of 0.12 M glutamate, 0.3 ml. of 0.2 M oxalacetate; incubated 5 or 30 minutes under nitrogen at 37°.

TABLE II

*Transaminase Activity of Cells and Cell-Free Enzyme*

Conditions as in Table III; incubation 5 minutes.

Preparation	Bacterial nitrogen	Aspartate formed	Transamination	$Q_T$ (N)
	mg.	microliters	per cent	
20 mg. dried cells	2.68	234	17.8	1,047
Cell-free enzyme from 20 mg.				
1.0 ml.	1.36	650	49	5,700
0.5 "	0.68	555	44	9,800
0.2 "	0.27	235	19	10,500

do not appear in the reaction as written. By using shorter incubation periods, 5 to 10 minutes, the side reactions appear to be largely eliminated.

The data in Fig. 3 indicate the effect of pH on the glutamic-aspartic transaminase enzyme of *Streptococcus faecalis* R. Both dried cells and cell-free transaminase preparations were used, and the rate of reaction found to be only slightly dependent on the pH within the range of 5.7 to 9.5. The relatively sharp optimum in the alkaline range as reported for animal tissue (11), plant cells (18), and *Escherichia coli* (14) is not apparent

for the *Streptococcus faecalis* preparations. The data for the latter resemble those reported by Cohen (12) and by Kritzmman (11) for purified transamination preparations.

TABLE III

Function of Pyridoxal Phosphate in Transamination with Dried Preparations of *Streptococcus faecalis* R

10 mg. of cell preparation, 2 ml. of 0.1 M phosphate buffer, pH 8.3, 0.5 ml. of 0.12 M glutamate, 0.3 ml. of 0.2 M oxalacetate; synthetic pyridoxal phosphate or pyridoxal as indicated; incubation at 37° under nitrogen.

Preparation	Glutamate used	Aspartate formed	Transamination*	Q <sub>T</sub> *
5 min. incubation				
Grown with pyridoxal	95	84	7	110
" without pyridoxal	25	9	1	20
+ 10 γ pyridoxal phosphate†	128	94	8	135
+ 50 " " .	107	86	7	115
Grown with pyridoxal	117	123	10	145
" without pyridoxal	0	0	0	0
+ 12 γ pyridoxal phosphate	101	137	10	140
+ 10 " " .	57	109	7	100
30 min. incubation				
Grown with pyridoxal	322		25	64
" without pyridoxal	136		11	27
+ 4 γ pyridoxal phosphate	186		15	33
+ 20 " " + 1 mg. adenosine tri-phosphate	223		17	45
Grown without pyridoxal	0		0	0
+ 15 γ pyridoxal phosphate	131		10	26
+ 1500 γ pyridoxal phosphate‡	287	358	24	32

\* Average of glutamate and aspartate values.

† Pyridoxal phosphate is expressed in pyridoxal equivalents as estimated by tyrosine decarboxylase assay (8).

‡ 20 mg. of cells

The cell-free transaminase, for which data are shown in Table II, was prepared by autolysis, as indicated under "Methods." For the particular preparation used in this case, 100 mg. of the dried cell preparation were

incubated in 5 ml. of 0.1 M phosphate buffer, pH 8.3, for 24 hours at 37° in the presence of toluene. After autolysis the suspension was centrifuged, to remove the bulk of the débris, and the supernatant cleared in a Beams ultracentrifuge. The resulting solution, which was almost clear, contained considerable transaminase activity. Aging the solution for 24 hours in the refrigerator caused more protein to precipitate. This was removed with the Beams ultracentrifuge to yield the water-clear supernatant, for which the data are given in Table II. The activity of this preparation on a protein basis ( $Q_T$  (N) 10,500) compares favorably with the purified transaminase which Cohen (12) isolated from animal tissue. The bacterial transaminase, however, is far from pure.

*Function of Pyridoxal Phosphate in Transamination*—Two methods of approach have been used to determine whether pyridoxal phosphate functions as the coenzyme of transamination. First, cells grown in a pyridoxal-deficient medium containing an excess of alanine were tested for transaminase activity alone and with added pyridoxal phosphate. The transaminase activity of cells grown in the same medium with added pyridoxal was used for comparison. Second, the cell-free transaminase enzyme was partially resolved to yield apoenzyme, and the ability of pyridoxal phosphate to restore activity determined.

Cells harvested from a medium deficient in pyridoxal and dried preparations from them were almost devoid of transaminase activity for the glutamate-aspartate system (Table III). The addition of synthetic pyridoxal phosphate to these cell preparations stimulates the transamination rate to a level comparable with that obtained for cells harvested from the same medium with excess pyridoxal present during growth. The stimulations were consistent and reproducible. In addition, there was usually good agreement between the glutamic acid disappearance and the aspartic acid formation. The stimulation of the transamination reaction with pyridoxal in the absence of adenosine triphosphate is in contrast to the results obtained with the tyrosine decarboxylase preparations (6). This is very probably due to the presence of appreciable quantities of adenosine triphosphate in those cells harvested from the neutral medium (see, for example, O'Kane and Umbreit (19)).

Attempts to resolve the cell-free transaminase by the methods used to resolve the amino acid decarboxylases (7, 8) were partially successful. Simple dialysis was not effective in removing the coenzyme, but aging coupled with dialysis did accomplish some resolution, apparently through the destruction of the coenzyme rather than by dissociation. The data in Table IV show that a variety of treatments resulted in varying degrees of resolution, and that the activity could be restored with pyridoxal phosphate. Pyridoxal was also effective in some cases, especially in the presence of

adenosine triphosphate, as was found to be the case with the tyrosine decarboxylase apoenzyme obtained by resolution of the enzyme from cells grown with pyridoxal (2, 8).

TABLE IV

*Function of Pyridoxal Phosphate in Transamination with Cell-Free Transaminase Conditions as for Table III.*

Preparation	Additions	Glutamate used	Aspartate formed	Stimulation
5 min. incubation				
Autolysate held 21 days and centrifuged in Beams ultracentrifuge	None	40	45	
	10 $\gamma$ pyridoxal phosphate	179	189	320
	21 " "	151	145	220
Autolysate dialyzed and held at 5° 11 days	None	97	73	
	10 $\gamma$ pyridoxal phosphate	121	136	50
Same, held 13 days	None	94		
	10 $\gamma$ pyridoxal phosphate	166		77
	25 " " + 1 mg. adenosine triphosphate	175		85
Autolysate held 9 days, 5°	None	200		
	12 $\gamma$ pyridoxal phosphate	240		20
Same, dialyzed 20 hrs.	None	48		
	12 $\gamma$ pyridoxal phosphate	104		110
30 min. incubation				
Autolysate held 6 days, then dialyzed and held 4 days at 5°	None	289		
	3 $\gamma$ pyridoxal phosphate	358		24
Autolysate held 6 days	None	238		
	15 $\gamma$ pyridoxal phosphate	393		63
	50 " " + 1 mg. adenosine triphosphate	289		25

## SUMMARY

Conditions for growing cells of *Streptococcus faecalis* R with highly active transaminase enzymes have been devised and the enzymes have been obtained in dried cell preparations and in a water-clear, cell-free condition.

Pyridoxal phosphate, previously shown to function as the coenzyme of amino acid decarboxylases, has been shown to function as the coenzyme of the glutamate-aspartate transaminase. This has been accomplished by two methods: (1) by growing cells in a medium deficient in pyridoxal to yield transaminase apoenzyme and activating the enzyme with synthetic pyridoxal phosphate; (2) by resolving the cell-free enzyme from organisms grown with pyridoxal and restoring the activity of this apoenzyme with pyridoxal phosphate.

The function of the vitamin B<sub>6</sub> group in protein metabolism is therefore at least partially explained by its action in amino acid decarboxylation and in transamination.

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# IDENTIFICATION OF SMALL AMOUNTS OF ORGANIC COMPOUNDS BY DISTRIBUTION STUDIES

## III. THE USE OF BUFFERS IN COUNTER-CURRENT DISTRIBUTION\*

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ELWOOD TITUS

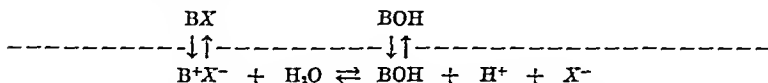
(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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In attempts to separate mixtures by "counter-current distribution" (1), or any other differential extraction procedure, it is obvious that a combination of immiscible solvents should be chosen which gives the greatest difference in the effective partition coefficients of the substances present in the mixture. This allows satisfactory separation to be made with the minimum number of transfers or plates, and saves much time and labor. The literature (2), as well as our own preliminary experience, is somewhat discouraging on this point and would seem to indicate in general that the ratios of the simple partition coefficients of two closely related substances are approximately the same in different combinations of solvents, although Synge (3) noted differences with acetylated amino acids.

However, a suggestion as to the most promising and versatile combination of immiscible solvents for use in separating organic acids or bases in small scale work is to be found in the first paper of this series (4) where the experience is given with partition coefficients of atabrine and closely related substances in the combination of an organic phase with a buffered aqueous phase. In this study such a partition coefficient was found to be a characteristic physical constant definitely useful in identification.

In the case of bases, the resultant equilibria involved in the measured partition coefficient may be represented schematically as follows, association being excluded:<sup>1</sup>



It has been shown previously (1) that the mathematical basis of counter-current distribution depends upon the percentage of the constituent which

\*The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research.

<sup>1</sup>An excellent treatment of acid-base equilibrium in simple two phase systems has been given by Murray (5) who suggested partition with adjustment of pH as a particularly good method for the separation of acids. King (6) has also made good use of the principle.

is transferred at each stage or plate, and since the procedure depends upon equilibration and progressive transfer from tube to tube, this percentage would be the total found in the phase moved. Thus the effective partition coefficient may include ionized and non-ionized forms, monomers, dimers, etc., where these forms are all in reversible equilibrium.

Accordingly, from the equilibria represented for bases, it is obvious that the effective partition coefficient is the resultant of at least two separate partition coefficients and the hydrolysis equilibria in the aqueous phase of the salt as well, with still other complications entering if  $HX$ , where  $X$  is the anion of the buffer, is appreciably soluble in the organic phase. If the simplest case is assumed in which the partition coefficient of the salt  $BX$  is vanishingly small, then the effective partition coefficient is the resultant of only two equilibria and this would usually be the most desirable system for practical use. In any case, it is apparent that an effective partition coefficient in the region of 1 for almost any base can be experimentally reached by the use of the proper buffer even though the free base has a very large organic phase-aqueous phase partition coefficient.

The type of equilibria represented would naturally be much less complicated in very dilute solution, and it is to be expected that the measured partition coefficient would not show as much shift with changes in the amount of total base in the two layers. However, if the method of counter-current distribution is to be used for the separation of mixtures, it is of considerable interest to know the upper limit of the amount of base which can be added to a fixed volume of the two phases before unpredictable events occur. Such information would permit the largest amount of base to be fractionated with the minimum volume of the two phases chosen.

This point was studied first with phosphate buffer, since this buffer appeared to be found in appreciable amounts in the organic phase only with those solvents which contained a considerable amount of water, such as the lower alcohols. Though it seemed desirable from a number of standpoints to study the most concentrated buffer which would not crystallize in the counter-current distribution machine at about  $26^\circ$ , a preliminary study of the effect of dilution of buffer was made. A typical result is recorded in Fig. 1, which shows the effect of dilution of the buffer on the partition coefficient of the antimalarial drug, plasmochin (isopropyl ether-phosphate buffer, pH 5.00, at 2 moles). A total of approximately 5 mg. of base was distributed between 10 cc. of each phase at  $26^\circ$ . The amount in each layer was determined spectroscopically by means of the Beckman quartz spectrophotometer.

A buffer approximately 2 M with respect to total phosphate ion was found to be a practical choice and could be prevented from crystallizing

over the entire buffer range by the appropriate combination of sodium and potassium ion. A high salt concentration should tend to give a larger partition coefficient of un-ionized base and thus perhaps a more delicate and specific over-all balance. Buffers were accordingly prepared so that

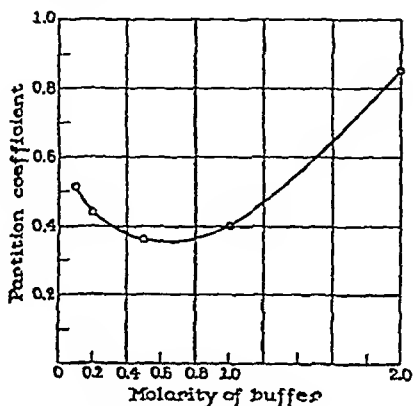


FIG. 1. Shift of partition coefficient with dilution of buffer

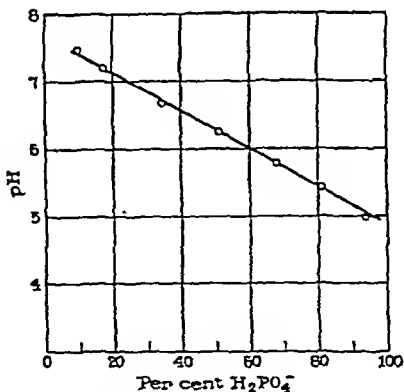


FIG. 2. Shift of pH with change of per cent  $\text{H}_2\text{PO}_4^-$  in the buffer

any desired pH within the range could be obtained by simple combination without appreciable change of total phosphate concentration. The change of pH with  $\text{H}_2\text{PO}_4^-$  ion as measured with the glass electrode against 0.1 M phthalate is shown in Fig. 2.



A major point of interest in the use of such a concentrated buffer system is precise information in regard to the amount of a given base which can be distributed in a given volume of each phase with predictable result. The shift of partition coefficient with total amount of base was therefore studied with a number of bases of the plasmochin type. Fig. 3 shows the shift of partition coefficient with different weights of base for the drug plasmochin when isopropyl ether and buffer (phosphate, pH 5.00) are the two phases. The volume of each phase was 10 cc. The two phases were shaken at room temperature (26°) and the amount in each phase determined spectroscopically. No attempt was made to obtain the highest accuracy, such as the use of a constant temperature bath, etc., would afford.

As can be seen from the data, the partition coefficient shifts greatly for amounts of base greater than 1 mg. per cc. of each phase. Moreover, at the higher concentrations of the base it was found much more difficult to check consecutive partition coefficient determinations, perhaps owing to small changes in temperature or of other conditions. Closely related bases of the same series were found to behave similarly and it was concluded from this preliminary experience that a range below approximately 1 mg. of base per cc. of each phase represented a more stable range in which to work than when more of the base was present. The highest concentration in Fig. 3 probably represents a case in which the buffer capacity has been exceeded, since the pH of the lower phase was found to be 5.36.

The cause of the shift of the partition coefficient with concentration except in the highest concentration was found not to be due to appreciable change in pH of the buffer upon addition of the base. It would seem probable that the shift is due to the tendency of the base to be associated in one or both of the phases, perhaps through hydrogen bonding. Such association could greatly shift the partition coefficient, as indeed was found here. This point of theoretical interest will be studied much more in detail as the opportunity presents itself.

A large shift in partition coefficient with variable amount of base would be expected to give a regularly skewed curve in the counter-current distribution apparatus instead of the symmetrical curve of error ordinarily expected with a pure substance (1). Fig. 4, Curve 1, shows this effect for plasmochin when a total of 80 mg. of the base is distributed, and Curve 2 when 10 mg. are distributed. The calculated theoretical curves are also given.

In actual practice with an unknown preparation it is easy to determine whether or not such deviation from the theoretical is due to impurity by simply determining the partition coefficient on either side of the maximum

at the most sensitive portion of the curve where any divergence is likely to be most noticeable. In Curve 1, Fig. 4, this would be in the region of about Tubes 7 and 21. If the partition coefficients of the two prove to be the same, the deviation may be regarded as due to some other cause than inhomogeneity.

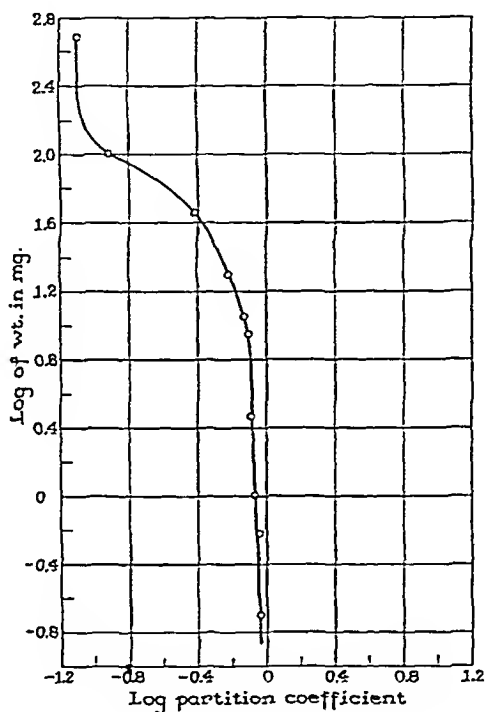


FIG. 3. Shift of partition coefficient with change of concentration (10 cc. of each phase).

Another point of considerable interest in the use of buffers is the degree to which the partition coefficient is shifted with change of pH, other factors remaining constant. The effect to be expected is shown very well with a study made on the free base of atabrine and of plasmochin, Fig. 5, in phosphate and citrate buffers.

From these curves it can be calculated that a shift of 0.02 pH unit will cause a shift of the partition coefficient from 1 to approximately 1.1, a change which would be plainly registered when the curve was determined

for a twenty-four plate distribution. When the distribution constant  $K$  is 1, the maximum would occur at 12 and for a  $K$  of 1.1 it would occur at 11.4. The twenty-four plate distribution is arbitrarily mentioned because the machines in current use in this laboratory contain twenty-five tubes instead of the twenty of the original model (1).

From these data and the data given in the first publication of this series (4) it is strikingly apparent that the partition coefficient of this type of system is a highly specific thing for the base concerned, in that even bases

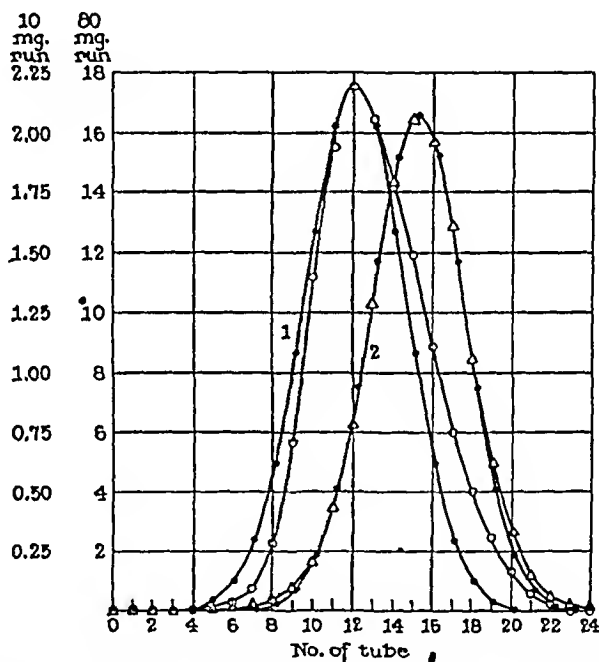


FIG. 4. Distribution curves for plasmochin.  $\circ$ , 80 mg. run;  $\Delta$ , 10 mg. run;  $\bullet$ , calculated values.

very closely related show considerable difference in their partition coefficients. When arbitrarily adjusted so that the constant is near 1, it obviously represents for the simplest case a delicate balance between the percentage of the un-ionized base found in the organic layer, a very large figure, relatively, and the per cent of ionized base to be found in the aqueous phase, a correspondingly small figure. The un-ionized base in the aqueous phase is in turn in equilibrium with a relatively large reservoir of base in the ionized form. The former balance is apparently shifted considerably for small differences in structure, while the latter figure could be shifted

considerably for small differences in basic strength. It is well known that small changes of structure in the vicinity of a basic nitrogen produce measurable shifts in the dissociation constant. It is also known that for slightly soluble substances a small change in structure frequently doubles or decreases by half or more that slight solubility. Thus, since in the partition coefficient ratios of large numbers over small, or the reverse, are involved, it is not surprising that the balance is a comparatively sensitive one.

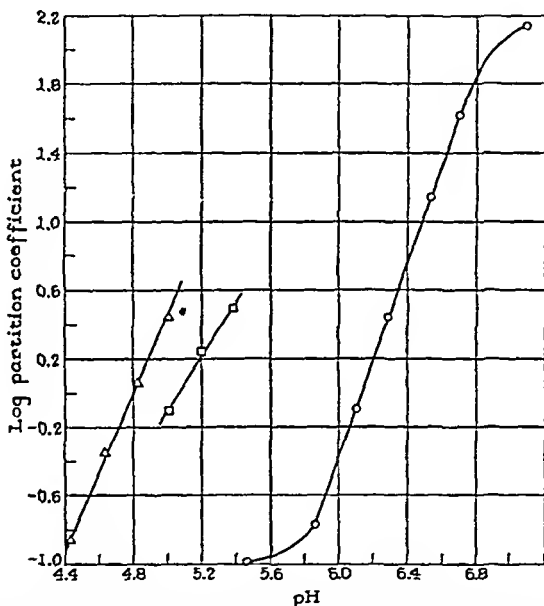


FIG. 5. Shift of partition coefficient with change of pH. O, atabrine in benzene-phosphate system; □, plasmochin in isopropyl ether-phosphate system; Δ, plasmochin in isopropyl ether-citrate system.

It was shown previously (1) that the rate of migration of the band of a homogeneous substance, that is the position of its maximum, can be calculated from Equation 1 at any time during operation, after a certain number of plates,  $n$ , have been applied.<sup>2</sup>

<sup>2</sup> In Equation 4 (1), giving the minimum number of plates required, a parenthesis has been misplaced. The equation should be

$$\left(1 - \frac{K}{K+1}\right)^n = 0.001$$

$$(1) \quad N = n \left( \frac{K}{K+1} \right)$$

The rate of migration is therefore directly proportional to  $K/(K+1)$ , the percentage which migrates forward during each step. Since the percentage which migrates forward is a ratio, with the integer 1 always added in the denominator, it follows that the maximum will be shifted most on the plotted curve for changes in  $K$  when the latter is nearest 1. Therefore it would appear desirable to adjust the system during empirical investigation so that the partition coefficient is as near 1 as possible in order that the smallest differences will have the largest apparent deviation from the theoretical on the plotted curve. From the equation describing the manner in which the band spreads out as it migrates, Equation 2 (1), it can also be derived that the band spreads most rapidly for the case when  $K$  is equal to 1.

$$(2) \quad \frac{dc}{dx} = \frac{C}{\sqrt{a\pi \left( \frac{K}{K+1} \right)^n}} e^{-x^2/a \left( \frac{K}{K+1} \right)^n}$$

Our experience thus far with buffers has plainly shown that for a great many mixtures the partition coefficients of the components are so widely removed from each other that it is not at all necessary to use a high number of plates or transfers to effect practically quantitative separation. When such is the case, the distribution machine is not essential, since the same thing may be accomplished with separatory funnels in a much shorter time and in a more flexible manner. Fractionation by the use of separatory funnels is an old familiar procedure with many variations. Perhaps the most efficient system of separating a two component mixture is that of multiple fractional distribution in separatory funnels, which was devised some time ago by Hunter and Nash (7).

However, when working with an unknown mixture by their method one lacks the precise information which would contribute to the best use of such a system, and, further, more than two components are present in the mixture more often than not. For empirical work many helpful suggestions can be derived from the view-point of counter-current distribution.

If Table I (1) showing the distribution to be expected is examined, when the effective partition coefficient is 1 it can be seen that for this one case the curve is always symmetrical, even for the first few transfers, and that in an eight plate distribution Tubes 0 and 8 will each contain only 0.4 per cent of the starting material. An eight plate distribution would therefore appear to be one which could be achieved in separatory funnels without too much trouble and would require a total of thirty-six separate shakings

and transfers, not counting those which are required to transfer the material to a single phase so that the total amount present in each tube can be quantitatively estimated. Such a complete operation has been found to require approximately 2 hours in this laboratory. Since an eight plate distribution has been found to be one which represents a compromise between the time and labor required, on the one hand, and a distribution sufficiently large, on the other, so that results can be interpreted by means of curves, it has been taken as a standard unit of procedure in this laboratory for preliminary empirical investigations.

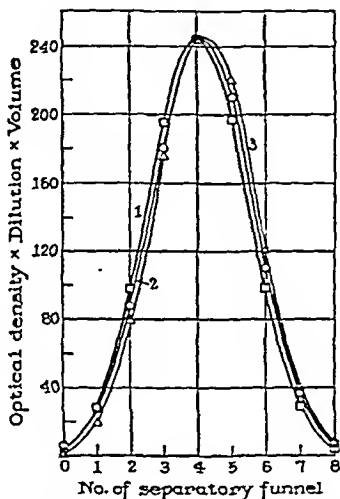


FIG. 6. Distribution curve for 6-methoxy-8-aminoquinoline. O, experimentally found curve; □, calculated curve,  $K = 1.0$ ; △, calculated curve,  $K = 0.9$ .

A theoretical eight plate distribution of a homogeneous substance,  $K = 1$ , would be that represented in Curve 1, Fig. 6; Curve 2 was obtained experimentally during an investigation with 6-methoxy-8-aminoquinoline in the system isopropyl ether-citrate buffer. The theoretical values for a distribution when the  $K$  is 0.9 are also given in Curve 3. As can be seen, the experimentally found values fall rather constantly between the two theoretical values. The details of the experimental procedure were as follows:

Nine separatory funnels, numbered Tubes 0 to 8, were arranged in a row and 10 cc. of the upper layer, previously saturated with the lower, were placed in each of the funnels. 10 cc. of the lower phase containing

the entire amount of the substance to be distributed were then added to Tube 0. After the tube was shaken and allowed to settle, the lower layer of Tube 0 was transferred to Tube 1 and 10 cc. of fresh lower layer were added to Tube 0. Both tubes were then shaken and the lower layer of Tube 1 transferred to Tube 2, while that of Tube 0 was transferred to Tube 1. Fresh lower layer was then added to Tube 0 and the process repeated until Tube 8 contained both layers, while Tube 0 contained only upper layer. The distribution was then complete and all that remained was to determine the total amount present in each separatory funnel, both upper and lower layers, so that it could be plotted as in Fig. 6.

This latter can be accomplished for bases by adding alkali to throw all of the substance into the upper layer, followed by estimation of the amount present by spectroscopic methods, by direct titration, or even by evaporating to dryness and weighing the residue. Numerous variations of this general procedure are obviously possible but the procedure given yields data at once comparable to those obtained with the distribution machine in which many more transfers are applied.

It will usually be found, after the data are plotted, that the balance will not be exact and that the effective partition coefficient will diverge somewhat from 1. A theoretical curve may be constructed, notwithstanding, by calculating the  $K$  from the position of the maximum and referring to a series of tables such as that given when  $K = 1$  (1). A series of such tables has been constructed for reference work in fitting curves of the values of  $K$  when equal to 0.9, 0.8, 0.7 · · · 0.1, and have been found very useful in this laboratory.

Soon after experience with this procedure in separating mixtures and a certain amount of practice in interpreting the curves so derived have been obtained, the significance of the partition coefficient of 1 will become somewhat more apparent. The method gives opportunity to select a single component at will by arbitrary adjustment of the system so that the maximum of this desired component will occur at the central tube, No. 4, whereas the undesired constituent will be thrown off in the end tubes in by far the largest majority of cases. Several such distributions run consecutively in different systems in their combined effect result in a method of considerable separating power for the purification of a substance or the resolution of a mixture.

Usually a method of analysis for determining the amount of substance present in each tube is not all inclusive and may not detect certain of the constituents. This is particularly true for spectroscopic methods in which certain constituents of unknown character may be transparent to the wave-length of light chosen. In this case consecutive distributions are particularly important, even though the curve so determined appears

that of a single substance. However, the probability of having a second band transparent to the method of analysis used, and also having a partition of exactly 1, is not very great. Consequently it may be detected in an apparently homogeneous curve by obtaining the weight of the substance present in Tubes 2 and 6 (or Tubes 1 and 7) to learn whether their extinction coefficients are the same. If they are not identical, a transparent band of foreign material would be suspected.

Determination of the partition coefficients of the material present in each of the tubes after distribution will also be found essential in the interpretation of results. Such coefficients should be determined on equal weights per unit volume of the two phases for each of the tubes so that an error will not be made owing to shift of partition coefficient with concentration or possible deviation from Beer's law when spectroscopic methods are employed.

Early in our study the possibility was considered of reaching the desired effective partition coefficient, that is, one giving 50 per cent of the total in each phase, by proportional adjustment of the volume of each phase, instead of changing the buffer. Such a practice was found to lead to *curves difficult to interpret when mixtures were concerned*. A pure substance with a coefficient of 2 will naturally give 50 per cent of the total in each phase if twice the volume of the lower phase is taken. This, however, does not necessarily follow for mixtures. One of the components might have a rather large coefficient and the second component might have a correspondingly small one, but the two exist in proportion so that the determined coefficient of the mixture could be 2. In such a case, adjustment of the volume of the lower phase to twice that of the upper would not give 50 per cent of the mixture in each phase as desired, but instead a value of the effective partition coefficient changed inappreciably from that of the determined value of 2 for exactly equal volumes. Naturally this is an exaggerated example without practical significance, but the same type of effect is present in diminished degree with ordinary mixtures which may be encountered.

#### SUMMARY

The advantages inherent in the use of buffers for the study of organic bases by the "counter-current distribution" technique have been investigated. Although certain limitations have been encountered, it is apparent that buffered systems offer a high degree of specificity when the proper working range is chosen.

Because of this specificity, a considerable degree of fractionation can usually be accomplished with relatively few transfers, and therefore the distribution may be carried out conveniently in ordinary separatory



funnels. A simplified procedure is given for nine separatory funnels in which the fractionation or distribution may be followed by the use of calculated theoretical curves.

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# AN AMINE FORMED BY BACTERIA DURING SULFONAMIDE BACTERIOSTASIS\*

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When *Escherichia coli* was grown in a synthetic medium containing salts, amino acids, and glucose in the presence of bacteriostatic concentrations of sulfonamides, an increase in total diazotizable amine was noted (1). This has now been found to be due to the production of a new heterocyclic amine, the nature of which may ultimately throw light on the mechanism of sulfonamide bacteriostasis.

The formation of the amine is not limited to one kind of bacterium nor to any specific sulfonamide drug. It was noted with a number of different organisms (Table I). Each of the drugs tried, sulfanilamide, sulfadiazine, sulfapyridine, and sulfathiazole, was found to be effective, but the actual amount formed varied with the concentration and nature of the drug. From the figures in Table II it is seen that the amount of new amine formed was not proportional to the amount of drug used, but the quantity formed was always of the same order of magnitude when bacteriostatic concentrations of different drugs were used. Since the ratio of amine formed to drug used was greatest with sulfadiazine, this drug was selected for preparation of the material for isolation.

*p*-Aminobenzoic acid, in concentrations sufficient to block the action of the sulfonamides, prevented the formation of the amine. None of the amine was formed in the absence of sulfonamides or when atabrine, plasmochin, or penicillin was used in concentrations sufficient to inhibit the growth of the bacteria.

The cultures used for chemical studies and for isolation were chiefly those of *Escherichia coli* with 0.5 mg. per cent of sulfadiazine as the bacteriostatic agent. The newly formed amine, in contrast to sulfonamides, was not acetylated in neutral aqueous solution by acetic anhydride. It could thus be distinguished conveniently and quantitatively from the sulfonamide in solutions containing both of the amines. Quantitative measurements of diazotizable amine by the Bratton-Marshall method for sulfonamide analysis (2) showed that the total amount after incubation was equal to

\* This work was done under the terms of a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University.

the sum of the amount of drug initially present and the amount of diazotizable amine remaining after acetylation (Table III). Thus none of the sulfonamide disappeared during the incubation in which the new amine was formed.

The amine has been isolated in a pure state from bacterial culture medium. When precautions were taken to avoid oxidizing conditions,

TABLE I  
*Effect of Variation of Bacteria*

The production of the new amine was compared when various bacteria were used with sulfadiazine as the bacteriostatic agent.

Bacteria	Concentration of sulfadiazine	Apparent yield of diazo- tizable amine <sup>a</sup>
	mg. per cent	mg. per cent
<i>Proteus vulgaris</i> .....	0.67	0.80
	4.95	0.56
<i>Shigella dysenteriae</i> (Shiga).....	0.67	0.28
<i>Salmonella paratyphi</i> B.....	0.67	0.11
<i>Escherichia coli</i> .....	4.95	1.00
<i>Eberthella typhosa</i> .....	4.95	1.02
<i>Vibrio comma</i> .....	4.95	0.26
<i>Shigella sonnei</i> .....	4.95	0.56
<i>Hemophilus influenzae</i> .....	1.25	1.54
	4.95	1.60
" <i>pertussis</i> .....	4.95	0.22
<i>Shigella paradysenteriae</i> .....	4.95	0.23
<i>Aerobacter aerogenes</i> .....	4.95	0.52

\* Measured by diazotization after acetylation of the amino group of the drug. The quantity of diazotizable amine was measured in all cases by the Bratton-Marshall method of sulfonamide determination (2) and the color read on a Coleman universal spectrophotometer at  $\lambda$  540 m $\mu$ . For convenience in calculation and comparison it was assumed that the molecular weight of the unknown amine was the same as that of sulfadiazine and that the compounds gave mole for mole the same amount of color on being diazotized and coupled.

a total of about 2 gm. of a pierate of the amine was obtained from about 200 liters of culture. By essentially the same isolation procedure, starting with a culture in which sulfapyridine replaced sulfadiazine as the bacteriostatic agent, the identical pierate was obtained. The nature of the amine does not depend upon the substituent on the amide group of the sulfonamide.

The pierate gives analytical values for the formula  $C_4H_6N_4O \cdot C_6H_5N_3O_7 \cdot 2H_2O$ . From it a number of other derivatives have been prepared, none of which is as stable as the pierate. Analyses of the free amine with and without water of crystallization are in good agreement with the empirical

TABLE II

*Effect of Variation of Bacteriostatic Agent*

The production of the new amine was compared when various concentrations of different sulfonamides served as the bacteriostatic agent

Drug	Concentration of drug	Increase of diazotizable amine*	Per cent increase
	<i>mg per cent</i>	<i>mg per cent</i>	
Sulfanilamide	35.0	0.78	2.2
	4.77	0.27	5.7
Sulfapyridine	4.98	0.54	11
	4.62	0.77	17
	2.49	0.49	20
	0.97	0.40	41
	4.9	1.05	21
Sulfathiazole	4.0	1.13	28
	3.1	1.13	36
	2.4	0.99	41
	2.0	0.74	37
	1.5	0.81	54
	1.0	0.57	57
	0.55	0.56	102
	0.92	1.27	138
Sulfadiazine	0.57	1.27	223
	0.45	1.14	253

\* Measured by diazotization after acetylation of the amino groups of the drug. See the foot-note to Table I.

TABLE III

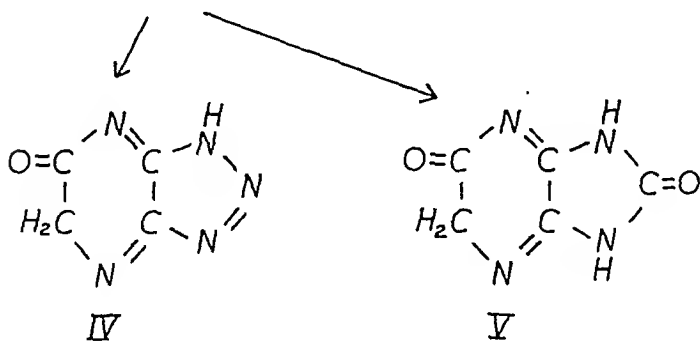
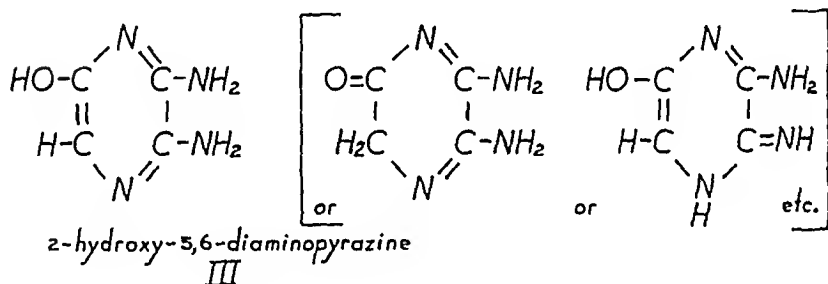
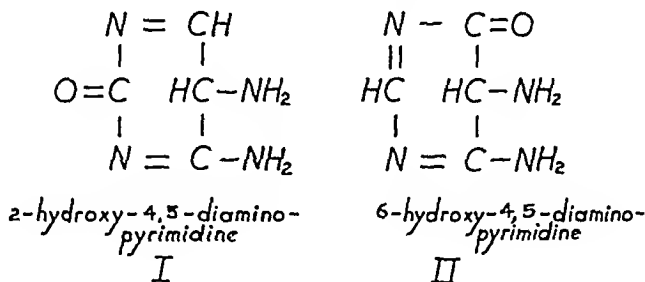
*Concentration of Non-Acetylated Amine*

The increase in the concentration of total diazotizable substances after incubation has been compared with the concentration of the non-acetylated component. In this experiment all samples were diluted in the same way for analysis and the colorimeter readings were converted to concentrations on the basis of a calibration curve

Drug	Concentration of drug	Total concentration of diazotizable amine after incubation	Increase in diazotizable amine during incubation (b - c)	Concentration of non acetylated diazotizable amine
	<i>(a)</i>	<i>(b)</i>		
	<i>mg per cent</i>	<i>mg per cent</i>	<i>mg per cent</i>	<i>mg per cent</i>
Sulfadiazine	0.92	2.20	1.28	1.27
	0.45	1.61	1.16	1.14
	0.52	1.25	0.73	0.69
Sulfapyridine	4.98	5.49	0.51	0.54
	2.49	2.97	0.48	0.49

\* See the foot-note to Table I.

formulae  $C_4H_6N_4O \cdot H_2O$  and  $C_4H_6N_4O$ . Samples of a chloroplatinate,  $(C_4H_6N_4O)_2 \cdot H_2PtCl_6 \cdot HCl$ , a sulfate,  $C_4H_6N_4O \cdot H_2SO_4$ , and a monohydrochloride,  $C_4H_6N_4O \cdot HCl$ , have also been prepared. The amine probably also forms a dihydrochloride which has not been obtained free of the



monohydrochloride. Although it is not acetylated in aqueous solution, the amine can be acetylated by acetic anhydride in glacial acetic acid.

The coupling ability of the diazotized solution diminishes on standing, on being heated, or when more concentrated nitrite or acid is used. The disappearance of the diazonium compound was not accompanied by

liberation of nitrogen. The product of prolonged action of nitrite was isolated; the empirical formula of  $C_4H_5N_3O \cdot H_2O$  suggested that cyclization to a triazole (IV) had occurred. The related assumption that our compound is an orthodiamine was supported by the ease with which the amine undergoes oxidative deamination and by the formation of a compound,  $C_6H_4N_4O_2$  (V), on fusion with urea, a reaction that has been used to differentiate ortho- from other diamines in the pyrimidine series (3, 4). An attempt to prepare a derivative with phenanthraquinone was unsuccessful.

An indication that the oxygen is phenolic was obtained by observing that the amine could be coupled with diazobenzenesulfonic acid to give an orange-yellow color in alkaline solution.

2 of the 4 nitrogen atoms in the compound appear to form part of a ring structure. From its empirical formula and many of its chemical properties the compound was at first suspected to be a monohydroxy-diaminopyrimidine. Of the various hydroxydiaminopyrimidines (5) only I and II, which contain ortho amino groups, come into consideration. 2-Hydroxy-4,5-diaminopyrimidine (I) which resembles our product in several respects (6, 7), was found to differ from it in that it decomposes at about  $265^\circ$  instead of melting at  $170^\circ$ , does not give the characteristic red color on diazotization and coupling, and shows an entirely different absorption curve in the ultraviolet (Fig. 1). Johns (7) described the formation of isobarbituric acid from 2-hydroxy-4,5-diaminopyrimidine when it was heated at  $140-150^\circ$  in a bomb tube with 20 per cent  $H_2SO_4$ , whereas our bacterial product gave glycine under similar conditions. The urea fusion product, 2,8-dihydroxypurine, prepared from 2-hydroxy-4,5-diaminopyrimidine gave a positive murexide test but an entirely different ultraviolet absorption curve from that of the corresponding derivative of our amine.

6,8-Dihydroxypurine, the product obtained by fusion of 6-hydroxy-4,5-diaminopyrimidine (II) with urea, crystallizes in long, narrow, shiny leaves and does not give a positive murexide test (8), whereas our product precipitates as a powder and gives a positive murexide test, carried out according to Fischer. A sample of 6-hydroxy-4,5-diaminopyrimidine itself, which has recently been synthesized (9), was found to give no color on being diazotized and coupled and to have a very different melting point from that of our amine.<sup>1</sup>

By vigorous hydrolysis with concentrated HCl in a bomb tube at  $150^\circ$  the ring of our amine was split and good yields of  $NH_3$  and glycine recovered. This fact we believe to contraindicate meta- and orthodiazine formulation, and to suggest that the ring structure is that of a paradiazine,

<sup>1</sup> We wish to thank Dr. R. O. Roblin, Jr., for this sample.

a pyrazine. We believe the most probable constitution to be the unknown 2-hydroxy-5,6-diaminopyrazine (III). This compound can readily be pictured as giving glycine and  $\text{NH}_3$  in good yields on acid hydrolysis and its properties would resemble those of the isolated amine.

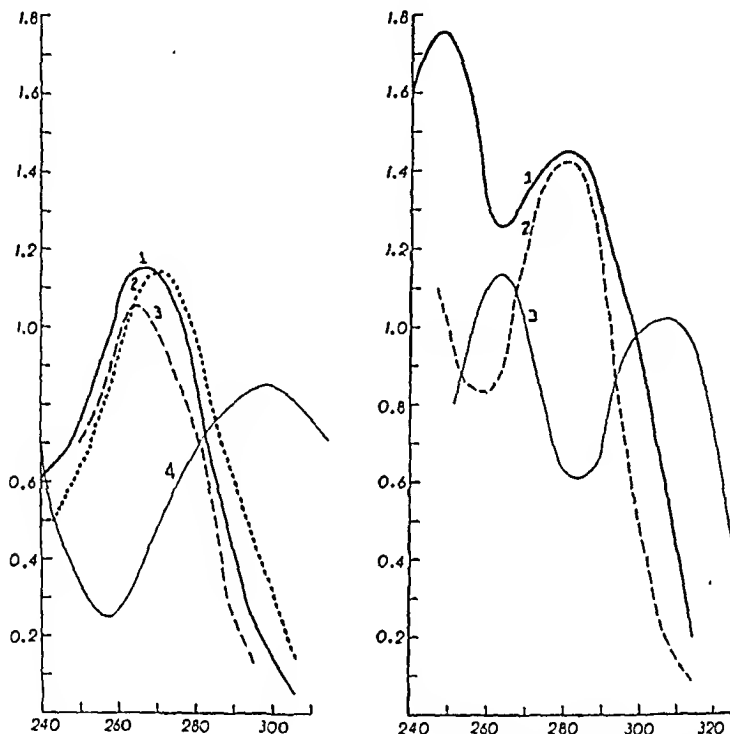


FIG. 1. Ultraviolet absorption spectra. The ordinate scale represents readings in  $\log I_0/I$  (1 cm.); the abscissa, wave-length in  $m\mu$ . Left-hand section, from isolated amine, 1.17 mg. per cent, at pH 7 (Curve 1), at pH 11 (Curve 2), at pH 3 (Curve 3); 2-hydroxy-4,5-diaminopyrimidine, 1.40 mg. per cent in dilute acid (Curve 4). Right-hand section, from isolated amine, 1.27 mg. per cent of triazole (Curve 1), 2.28 mg. per cent of imidazolone, pH 11 (Curve 2), 1.93 mg. per cent of 2,8-dihydroxypurine, pH 11 (Curve 3).

A number of as yet unsuccessful attempts have been made to synthesize this compound or its urea fusion product. The only approach which has yielded any encouraging results has been the condensation of cyanogen with glycinamide, analogous to the formation of diaminobenzpyrazine from *o*-phenylenediamine (10). Treatment of glycinamide in a water-alcohol solution with cyanogen gave among other products minute amounts of a product which behaved like our isolated amine with regard to the

diazo test and the difficulty of acetylation. Unfortunately it has not yet been found possible to isolate this product in a pure state.

The biological rôle of the product is as yet obscure. In tests carried out so far the compound fails to exhibit striking effects on bacterial growth. It is not removed from the medium by normally growing *Escherichia coli*.

Rings of the paradiazine type are not unknown in nature, and in some instances occur in substances of striking biological activity. They are found in such condensed ring systems as alloxazine and in the pterins, which may be related to "folic acid"<sup>2</sup> (11). Dutcher and Wintersteiner (12) have recently shown that aspergillie acid is probably a substituted pyrazine.

The isolation of what is probably 2-hydroxy-5,6-diaminopyrazine from culture media in which the normal proliferation of bacteria has been prevented by addition of sulfonamides suggests a hitherto unsuspected type of action of this group of bacteriostatic agents. As the product does not arise directly from the sulfonamide used, it must be pictured as having been produced by action of the bacteria on other constituents of the medium. It may be either a normal intermediate in some metabolic reaction which is blocked by the drug or an abnormal product formed by the bacteria under the influence of the drug.

#### EXPERIMENTAL

*Production of the Amine*<sup>3</sup>—The basal medium used was a modified Long's medium containing in each liter 5.0 gm. of  $\text{NH}_4$  citrate, 3.0 gm. of  $\text{KH}_2\text{PO}_4$ , 3 gm. of  $\text{Na}_2\text{CO}_3$ , 2 gm. of  $\text{NaCl}$ , 0.2 gm. of  $\text{MgSO}_4$ , 0.05 gm. of ferric ammonium citrate, 0.005 gm. of nicotinic acid, 2 gm. of glucose, and 10 cc. of a 5 per cent solution of a hydrolysate of either gelatin or casein. The effects of various concentrations of different sulfonamides are given in Table II.

Using *Escherichia coli*, we observed that the yield was increased by expanding the surface area exposed to the air, but was greatly decreased when air was slowly bubbled through the growing culture. Substitution of equivalent amounts of pyruvate, lactate, or succinate for the glucose

<sup>2</sup> We wish to thank Dr. W. H. Sebrell of the National Institute of Health for his assay of our compound for "folic acid" activity. He reported: "The results of the 'folic acid' assay of your compound are as follows: Rat assay, no activity at 250  $\gamma$  per rat per day (5  $\gamma$  of 'folic acid' would give a positive test). *S. lactis* R, tested in concentrations up to 560  $\gamma$  per tube. Less activity than  $2.5 \times 10^{-4}$   $\gamma$  of 'folic acid.' *L. casei*, tested in concentration up to 210  $\gamma$  per tube. Less activity than  $3 \times 10^{-4}$   $\gamma$  of 'folic acid.' In view of these results, I think we can say that the compound has no 'folic acid' activity."

<sup>3</sup> The authors wish to thank Mr. Saul Frances for assisting with some of the bacteriological work.



of the medium resulted in increments of 27, 5, and 7 per cent respectively as compared with about 200 per cent when glucose was used.

Inocula of 1 to 3 million *Escherichia coli* per cc. of medium were found to produce satisfactory amounts of the amine. A 10-fold increase of inoculum resulted in no increased amine formation, while reduction of the inoculum to one-tenth greatly decreased the yield. 18 hours were found to be the optimum time of incubation.

In addition to *Escherichia coli* a number of other bacteria which could be grown in our media were tested, and the production of diazotizable amine in the presence of sulfonamide noted. The medium used in these experiments consisted of the usual basal medium containing casein hydrolysate and glucose, to each liter of which were added 50 mg. of cysteine hydrochloride and 0.2 mg. of thiamine hydrochloride. Each solution was inoculated with 1 million bacteria per cc. and incubated for 18 hours at 37°. The bacteria were then removed and the quantity of diazotizable amine was measured before and after incubation and after acetylation of the incubated solution. The results are given in Table I.

Strains of sulfonamide-fast *Staphylococcus aureus* (F<sub>7</sub> and F<sub>14</sub> Landy) (13) were grown in our medium with added thiamine and cysteine with no drug present. The amine which accumulated, thought by Landy to be *p*-aminobenzoic acid, although diazotizable, was unlike our amine in being readily acetylated. It was noted that the specific soil bacillus used by Mirick to oxidize *p*-aminobenzoic acid (14) destroyed the amine formed by F<sub>7</sub> and F<sub>14</sub> but did not destroy our amine.<sup>4</sup>

The large scale cultures from which the amine was isolated were grown in 4 liter Pyrex glass bottles. 2 liters of culture medium containing 0.5 mg. per cent of sulfadiazine, but no glucose, were placed in each bottle and the solution adjusted so that after autoclaving the pH was between 7.0 and 7.6. 2 gm. of glucose per liter were added and the medium inoculated with 1 cc. of a 24 hour culture of *Escherichia coli* per liter, the resulting initial bacterial concentration being about 1 million bacteria per cc. The bottles were laid on their sides to increase the surface area and were incubated for 18 hours at 37°.

*Properties of Dilute Solutions of the Amine from Bacterial Cultures*—After removal of the bacteria from the incubated solution by means of a Berkefeld filter or a Sharples centrifuge, the properties of the amine in the resulting solution, containing about 1 mg. per cent of the amine, were studied. In this crude mixture the amine was relatively stable in boiling acidic, alkaline, or neutral solution. The amine was diazotizable and its

<sup>4</sup> We wish to thank Dr. M. Landy for providing the subcultures of his sulfonamide-resistant *Staphylococcus aureus* and Dr. G. S. Mirick for subcultures of his soil bacillus which were used in these experiments.

diazo derivative, when coupled with N-(1-naphthyl)ethylenediamine dihydrochloride, gave a colored compound which had nearly the same absorption maximum in the visible region as the corresponding products obtained from any of the sulfonamides. In contrast to the sulfonamides, the amine was not acetylated by 2 per cent acetic anhydride in neutral aqueous solutions. This property proved very useful as a means of quantitatively measuring the compound in the presence of the drug and of following its separation from the drug. In practice these determinations were made by the Bratton-Marshall method (2) before and after half an hour's treatment with 2 per cent acetic anhydride at room temperature, as described by Rosenthal (15). The diazotizable amino group of each of the sulfonamides was readily and completely blocked by acetylation, while that of the unknown amine was not blocked (Table III). Excellent agreement was obtained between the total amount after incubation and the sum of the initial amount of drug used plus the amount remaining after acetylation of the incubated solution. Thus the sulfonamide was not lost during the incubation period in which the new amine was formed. In order to get this agreement it was found necessary to dilute all samples in the same way and to calculate the concentrations from the colorimeter readings on the basis of a calibration curve.

Unlike sulfadiazine the amine was not removed from a water solution at pH 4 to 6 by continuous ether extraction. The amine could be removed from the dilute water solution slowly by continuous extraction with butanol or by extraction with ethanol of a water solution saturated with  $K_2CO_3$ . Its extraction with acetone from an aqueous solution saturated with  $CaCl_2$  was accompanied by considerable loss of material. Chloroform, benzene, and ethyl acetate did not extract the amine.

The amine in solution was apparently readily destroyed by oxidizing agents. Short exposure to  $H_2O_2$  in acidic, alkaline, or neutral solution or to  $Ag_2O$ ,  $Cl_2$ , or  $KMnO_4$  resulted in total loss of diazotizability. Even aeration resulted in gradual disappearance of diazotizable amine from solution. The diazotizable properties were unaffected by  $Zn$  and  $HCl$ .

No adsorption was observed on  $Al_2O_3$ ,  $MgO$ , Johns-Manville Standard Super-Cel,  $CaO$ , talc,  $SiO_2$ , or  $MgHPO_4$  when each of these was shaken with a neutral solution of the amine. The material disappeared from solution when shaken with charcoal but could not be eluted from the charcoal by acid, ammonia, or alcohol. It could be adsorbed on fullers' earth from acid solution and eluted with concentrated ammonia in 50 per cent alcohol.

It was found that the amine was not readily precipitated from the dilute solution by lead acetate,  $Ca(OH)_2$  and  $Cu(OH)_2$  or tannic acid, but was readily precipitated by phosphotungstic acid and by mercuric ion from a slightly alkaline solution.

*Isolation Procedure*—After removal of the bacteria the pH was adjusted to 7 to 7.5 with NaOH and a freshly prepared water solution containing 1 gm. of mercuric acetate was added to each liter. The flocculent white precipitate which immediately began to settle contained almost all of the amine as well as considerable  $\text{NH}_3$  and some amino acids. After the precipitate had settled overnight, the supernatant was discarded and the precipitate washed well several times with water by centrifugation. A relatively large volume of 2 N HCl was required to dissolve most of the mercury-amine compound. The solution became progressively deeper red-brown in color and a caramel-like odor was noted. A large amount of insoluble material was filtered off and the mercury was removed with  $\text{H}_2\text{S}$ . After removal of  $\text{H}_2\text{S}$  by boiling, the pH was adjusted to 4 to 6 and the solution was extracted continuously with peroxide-free ether<sup>5</sup> for 2 days to remove sulfadiazine and other ether-soluble impurities. All of the diazotizable amine in the ether solution after this procedure was acetyltable with acetic anhydride in neutral water solution, whereas nearly all of that remaining in the water solution after extraction was non-acetyltable under the same conditions.

The water solution was then evaporated at reduced pressure to a small volume in an atmosphere of nitrogen at room temperature. It was observed that whenever air was passed through the solution for any length of time the amine was decomposed. To remove ammonia the solution was made alkaline to phenolphthalein with  $\text{Ba}(\text{OH})_2$  and evaporated. After removal of the barium, the pH of the pale yellow solution was adjusted to 7 to 7.5 and the amine reprecipitated with mercuric acetate. This second mercury precipitation in the absence of carbonate effected a further separation of the amine from amino acids. The mercury was again removed, the acidic solution evaporated almost to dryness *in vacuo* under nitrogen, and the residue extracted several times with aldehyde-free ethanol. Most of the solvent was removed, the residue taken up in water, and a small amount of ethanolic picric acid solution added. The well formed yellow needles which precipitated from the solution after it had stood in the refrigerator overnight were filtered off, washed with cold water, and recrystallized twice from very dilute hot alcohol or from a larger volume of hot water. 78 mg. of the crystalline picrate were isolated from 25 liters of culture. From 82 liters of culture 1.0 gm. of picrate of the amine was obtained and in a third preparation 0.89 gm. of picrate was isolated from 67 liters of culture.

*Picrate*—An air-dried sample was dried at  $100^\circ$  *in vacuo* over  $\text{P}_2\text{O}_5$  and

<sup>5</sup> Due to the ease of oxidative destruction of the amine, all evaporations were carried out under nitrogen and all solvents were carefully freed of oxidizing contaminants.

lost 9.2 per cent of water. The theory for  $C_4H_6N_4O \cdot C_6H_3N_3O_7 \cdot 2H_2O$  is 9.2 per cent of water. Decomposition point  $240^\circ$ .



Theory.

C 33.8, H 2.53, N 27.6, picric acid 64.5

Found. 1st preparation.

" 33.1, " 2.82, " 26.3,\* S 0, picric acid 64.2

2nd

"

" 33.5, " 2.97, " 25.9

\* The microanalyses were performed by Mr. William Saschek. The nitrogen analyses were carried out by the standard Pregl micro-Dumas method and were in some cases somewhat lower than the theory for the formula which we propose. Some heterocyclic nitrogen compounds are known to give low values for nitrogen by this method (16).

Picric acid was determined gravimetrically by precipitation of nitron picrate (17).

*Amine Isolated from Culture in Which Sulfapyridine Was Used As Bacteriostatic Agent*—The compound was also isolated from cultures in which growth was inhibited by 6 mg. per cent of sulfapyridine instead of 0.5 mg. per cent of sulfadiazine, all other conditions being kept the same. The same method of isolation was followed, though several additional precipitations with mercuric acetate were necessitated by the larger relative amounts of the drug present. 0.2 gm. of picrate was obtained from 32 liters of culture. The picrate isolated was identical with that previously obtained. Found C 33.4, H 2.53, N 26.6.

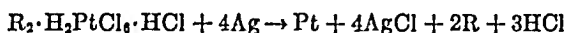
The ultraviolet absorption curve of the amine from this preparation was identical with that of the compound isolated from the cultures containing sulfadiazine (Fig. 1).

*Chloroplatinate*—165 mg. of anhydrous picrate were suspended in a small volume of concentrated HCl and the picric acid extracted with peroxide-free ether. After the solution had been evaporated to about 3 cc., 300 mg. of  $H_2PtCl_6 \cdot 6H_2O$  dissolved in a few drops of water were added to the warm concentrated acid solution of the amine. Shiny orange plates of the chloroplatinate of the amine precipitated out on cooling. The crystals were filtered cold, washed with a very small volume of concentrated HCl, then with a little cold water, and dried to constant weight *in vacuo* over  $P_2O_5$  and KOH at room temperature. 133 mg. were obtained. It was found that the chloroplatinate could not conveniently be recrystallized because the amine is a sufficiently strong reducing agent to precipitate metallic platinum from a hot water solution of the chloroplatinate. M.p.  $210-215^\circ$  with decomposition.

$(C_4H_6N_4O)_2 \cdot H_2PtCl_6 \cdot HCl$ . Theory. C 13.7, H 2.15, N 16.0, Pt 28.0, Cl 35.6

Found. " 13.8, " 2.33, " 15.3, " 27.9, " 33.5  
28.2

A sample of the chloroplatinate, after the removal of platinum with silver by the method of Dudley (18), gave a solution which on titration with NaOH was found to contain 3 equivalents of acid per mole of chloroplatinate.



*Isolation of Free Amine*—Picric acid was removed from 100 mg. of the picrate by ether extraction from a suspension in aqueous  $H_2SO_4$ . The bulk of the sulfate was removed with  $Ba(OH)_2$  and the slightly acidic filtrate evaporated to a volume of about 2 cc. The remaining sulfate was quantitatively removed with  $BaCO_3$  and the solution freed of water by repeated evaporation from alcohol-benzene. The residue was taken up in about 1 cc. of alcohol, a small amount of insoluble material was filtered off, and 5 cc. of benzene were added to the filtrate. The large colorless needles which formed on cooling were centrifuged off, washed with benzene, and recrystallized from alcohol-benzene. The recrystallized sample, weighing 8 mg., was dried *in vacuo* over  $P_2O_5$  without heat; m.p.  $168-170^\circ$  with decomposition.

$C_6H_8N_4O \cdot H_2O$ . Theory, C 33.3, H 5.6, N 38.9; found, C 33.3, H 5.4, N 38.7

A deep purple precipitate appeared in the mother liquor after several days and in a second preparation the entire sample was lost as a purple product.

Isolation of the free amine from a very small volume of water was found to be more convenient than from alcohol-benzene. A water solution of the amine, equivalent to 56 mg., prepared by removal of picric acid and sulfate as previously described, was carefully evaporated to a volume of less than 1 cc. The yellowish tan crystals when formed on cooling were washed with a small amount of cold water. The sample was recrystallized from 1 cc. of water without undue heating and small colorless needles were obtained. Yield, 18 mg.

*Water Determination*—11.53 mg. dried to constant weight at room temperature and atmospheric pressure over  $CaCl_2$  lost 1.39 mg. when dried at room temperature over  $P_2O_5$  *in vacuo*. When dried at  $110^\circ$  over  $P_2O_5$  *in vacuo*, only 0.09 mg. more was lost.

$C_6H_8N_4O \cdot H_2O$  Theory,  $H_2O$  12.5; found, 12.8

$C_6H_8N_4O$ . Theory, C 38.1, H 4.8, N 44.4; found, C 38.2, H 5.1, N 43.8

*Spot Tests*—A few drops of a solution of the free amine gave a positive biuret test with concentrated NaOH and dilute  $CuSO_4$ . In a short time the lavender color was masked by the formation of a black precipitate.

1 mg. of the amine gave a deep blue color with NaCN in dilute  $\text{NH}_4\text{OH}$  and lithium arsenotungstate reagent (19).

Negative results were obtained when murexide and Sakaguchi tests were carried out on solutions of the amine.

When the amine was treated with *p*-diazobenzenesulfonic acid in alkaline solution, a deep yellow-orange color appeared. Phenol similarly treated gave a deep yellow color, whereas no color was produced with either aniline or *o*-phenylenediamine.

*Hydrochloride*—Difficulties were encountered in the preparation of the amine hydrochloride. After removal of picric acid from a suspension of the picrate in HCl, a tan crystalline product could be obtained by concentration of the aqueous layer, by saturation with HCl gas, or by addition of alcohol. On analysis it appeared to be a mixture of the mono- and dihydrochlorides of the amine.

$\text{C}_6\text{H}_8\text{N}_4\text{O} \cdot \text{HCl}$ . Theory. Cl 22.2, N 34.4

$\text{C}_6\text{H}_8\text{N}_4\text{O} \cdot 2\text{HCl}$ . Theory, Cl 35.6, N 28.1; found, Cl 26.9, N 31.2

The monohydrochloride was finally purified by precipitation from a large volume of absolute alcohol by the addition of several volumes of benzene. The white needles which slowly appeared in the solvent mixture decomposed at 210–215°.

$\text{C}_6\text{H}_8\text{N}_4\text{O} \cdot \text{HCl}$ . Theory. C 29.5, H 4.3, N 34.4, Cl 22.2

Found. " 29.9, " 4.2, " 32.3, " 22.2

*Sulfate*—The sulfate, like the hydrochloride, proved difficult to obtain in pure form. After removal of picric acid from a suspension of the picrate in dilute  $\text{H}_2\text{SO}_4$ , the solution was evaporated to a small volume and the sulfate precipitated as an oil by the addition of alcohol-ether. After long cooling the oil solidified to a tan powder which appeared to deteriorate on attempted recrystallization.

$\text{C}_6\text{H}_8\text{N}_4\text{O} \cdot \text{H}_2\text{SO}_4$ . Theory, C 21.4, H 3.57; found, C 21.2, H 3.66

*Investigation of Properties of Amino Groups*—The intensity of color obtained when the amine was diazotized and coupled with *N*-(1-naphthyl)-ethylenediamine was greatly influenced by the conditions. The greatest amount of azo dye was obtained when the amine was dissolved in acetic acid and treated with dilute  $\text{NaNO}_2$ . When the conditions of the Bratton-Marshall method (2) of sulfonamide analysis, dilute HCl and dilute  $\text{NaNO}_2$ , were used, only slightly less color was obtained. Increasing the concentration of either the HCl or the  $\text{NaNO}_2$  resulted in appreciably less dye formation. A large portion of the diazonium salt disappeared on standing at room temperature for half an hour and the disappearance was almost com-

plete if the solution was heated for half an hour on the steam bath. No nitrogen was evolved when the amine was treated with 30 per cent  $\text{NaNO}_2$  and either glacial acetic acid or 3 N  $\text{HCl}$  and shaken for half an hour at room temperature in a Van Slyke apparatus (20). Under similar conditions 97 per cent of the diazonium salt had been shown colorimetrically to have disappeared.

*Condensation with Nitrous Acid; Triazole (IV)*—To a solution of 40 mg. of the amine in 10 cc. of 2 N  $\text{H}_2\text{SO}_4$ , 0.5 gm. of  $\text{Ba}(\text{NO}_2)_2$  dissolved in a small amount of water was added. After having stood overnight at room temperature the solution was found to contain only a trace of diazotizable amine. The solution was warmed on a steam bath for 5 minutes, hot  $\text{Ba}(\text{OH})_2$  solution added until a slight excess of sulfate remained, the solution made slightly alkaline with  $\text{NaOH}$ , and the  $\text{BaSO}_4$  filtered off. After acidification with  $\text{H}_2\text{SO}_4$  the filtrate was evaporated to a small volume and the pale yellow precipitate which formed on cooling was filtered and washed with a small amount of cold water. The precipitate was purified by solution in  $\text{NH}_4\text{OH}$  and acidification with  $\text{H}_2\text{SO}_4$ . The pale yellow rosettes<sup>6</sup> of needles which formed slowly on standing in the refrigerator were washed with cold water and dried *in vacuo* over  $\text{P}_2\text{O}_5$  at  $100^\circ$ . Yield, 27 mg.; the substance began to darken at about  $150^\circ$  and did not melt below  $260^\circ$ .

$\text{C}_4\text{H}_2\text{N}_6\text{O} \cdot \text{H}_2\text{O}$ . Theory, C 31.1, H 3.2, N 45.1; found, C 31.3, H 3.2, N 43.9

When a dilute solution of the triazole in  $\text{HCl}$  was treated with the usual coupling compound, no color appeared at first but a faint pink color developed gradually on standing. This color had the same absorption maximum in the visible region as did that obtained from the diazotization and coupling of the amine itself, suggesting reversal of the ring closure.

An unsuccessful attempt was made to prepare a phenanthraquinone derivative of the amine.

*Fusion with Urea; Imidazolone (V)*—A sample of the amine was fused with urea by a method used for converting *o*-diaminopyrimidines into purines (3, 4). A solution of 40 mg. of the amine in dilute  $\text{HCl}$  was neutralized with  $\text{NH}_4\text{OH}$ , the solvent evaporated, and 40 mg. of urea added. The dry mixture was heated at  $170$ – $180^\circ$  for 1 hour, during which time considerable ammonia was evolved. The residue was dissolved in 1 cc. of dilute  $\text{NaOH}$ , a small amount of slimy material filtered off, and the solution acidified with a drop of concentrated  $\text{HCl}$ . The white powder which precipitated was washed with water and purified twice by solution in

<sup>6</sup> It was subsequently found that the triazole could be obtained as white crystals after treatment of a hot water solution with charcoal, but insufficient material remained for analysis.

$\text{NH}_4\text{OH}$  and precipitation by acidification with  $\text{HCl}$ . 21 mg. were obtained. The product required drying *in vacuo* at  $135^\circ$  for many hours.

$\text{C}_5\text{H}_4\text{N}_4\text{O}_2$ . Theory, C 39.5, H 2.63, N 36.9; found, C 39.3, H 2.86, N 36.6

*Acetylation*—Even though the amino groups of our compound were not acetylated under the mild conditions sufficient for the acetylation of sulfonamides, it was found that they could be completely acetylated when more vigorous conditions were used. Several mg. of the amine in a drop of water were heated on a steam bath for 1 hour with 1 cc. of acetic anhydride. A portion of the resulting solution, when treated with nitrite and the usual coupling compound, gave no color, while another portion after being boiled for 5 minutes with dilute  $\text{HCl}$  and similarly diazotized and coupled gave a deep red color.

*Oxidative Degradations*—A sample of the amine in a dilute  $\text{Ba}(\text{OH})_2$  solution containing 2 per cent hydrogen peroxide was boiled gently for 1 hour. Three-fourths of the N initially present could be accounted for as  $\text{NH}_3$  evolved during the oxidation. The remaining solution was acidified and the  $\text{CO}_2$  collected in  $\text{Ba}(\text{OH})_2$ . The  $\text{BaCO}_3$  which formed accounted for about half of the C of the amine. In 20 per cent  $\text{NaOH}$  solution, peroxide converted all of the N of the amine into ammonia. No urea could be isolated from the solutions after oxidation with chlorine, alkaline  $\text{KMnO}_4$ , and chloroplatinic acid.

*Degradation to Glycine and Ammonia*—40 mg. of the amine in the form of the crude hydrochloride were heated in a sealed tube with 2 cc. of concentrated  $\text{HCl}$  for 20 hours at  $150^\circ$ . The resulting yellow solution was evaporated to a very small volume; 28 mg. of  $\text{NH}_4\text{Cl}$  (55 per cent of theoretical) were obtained.

The mother liquor was made barely alkaline with  $\text{Ca}(\text{OH})_2$  in an attempt to identify oxalic acid among the products, but no precipitate was formed. The solution was then made strongly alkaline with  $\text{NaOH}$  and  $\text{N}_2$  gas was passed through the warmed solution for about an hour to remove the remaining ammonia. The solution was stirred for 6 hours with 100 mg. of *p*-toluenesulfonyl chloride in ether. The alkaline solution was extracted with ether to remove excess reagent, acidified with  $\text{HCl}$ , and the *p*-toluenesulfonyl derivative extracted with ether and crystallized from water. 44 mg. of *p*-toluenesulfonylglycine were obtained (63 per cent of the theoretical). The product was recrystallized twice from water and dried *in vacuo* at  $61^\circ$  for analysis; m.p.  $147\text{--}149^\circ$ . A mixture of the compound with a sample of *p*-toluenesulfonylglycine showed no depression of melting point.

$\text{C}_8\text{H}_{11}\text{O}_4\text{NS}$ . Theory, N 6.11, S 13.97; found, N 6.09, S 13.80

*2-Hydroxy-4,5-diaminopyrimidine (I)*—A sample of 2-hydroxy-4,5-



diaminopyrimidine was prepared from cytosine<sup>7</sup> by nitration followed by reduction of the nitrocytosine (7). The yellowish needles, dried *in vacuo* over  $P_2O_5$  at room temperature, melted at about  $265^\circ$  with decomposition. On diazotization and coupling with N-(1-naphthyl)ethylenediamine no red color was formed. It was noted that similar treatment after a sample had been boiled with dilute HCl resulted in the appearance of a gradually intensifying pale pink-lavender color.

$C_4H_6N_4O$ . Theory, C 38.1, H 4.8; found, C 37.8, H 5.1

Some of I was fused with urea at  $180-190^\circ$  and 2,8-dihydroxypurine isolated (7). This purine gave a positive murexide test. The ultraviolet absorption curves of both the pyrimidine and the purine are given in Fig. 1.

*Attempted Syntheses of 2-Hydroxy-5,6-diaminopyrazine (III)*—A number of attempts to prepare III by condensation of glycineamide with oxamide were made. Successively chloroform, alcohol, anhydrous  $H_2SO_4$ , and acetic anhydride, both cold and hot, were tried unsuccessfully as media for the condensation.

The most promising methods to date were those starting with glycineamide and cyanogen. An exact equivalent of aqueous NaOH was added to a solution of glycineamide hydrochloride in ethanol and 2 times the theoretical amount of cyanogen, generated from KCN and  $CuSO_4$  (21), was passed into the solution. After a few minutes the solution became yellow-tan and then red-brown in color and a brown precipitate formed on standing. A few drops of the solution after diazotization and coupling gave a deep red color of the same intensity both before and after the solution had been treated with dilute acetic anhydride. From the intensity of color we have estimated that at best about 100 mg. of the amine were formed when 5 gm. of glycineamide were used. When cyanogen was passed into a water-alcohol solution containing no glycineamide, the same red-brown color and precipitate were noted but the solution contained no diazotizable amine. About the same small amount of diazotizable amine was formed when either water or ethanol was used as the solvent. When methanol was used instead, less was produced and still less was formed when cyanogen was dissolved in  $CH_3OH$  prior to addition of the glycineamide. No diazotizable amine was produced with chloroform, dilute HCl, dilute NaOH, or  $NH_4OH$  as the solvent.

#### SUMMARY

When the normal growth of certain bacteria is inhibited by bacteriostatic concentrations of sulfonamide drugs, a diazotizable amine is found to accumulate in the medium.

<sup>7</sup> We wish to thank Professor T. B. Johnson for the sample of cytosine used in this synthesis.

From cultures of *Escherichia coli*, whose growth was inhibited by either sulfadiazine or sulfapyridine, the amine has been isolated as a picrate and samples of the free amine and a number of derivatives prepared. It is a heterocyclic orthodiamine having the empirical formula  $C_4H_6N_4O$ . The possibility of its being a pyrimidine has been eliminated. Its properties indicate that it is probably 2-hydroxy-5,6-diaminopyrazine.

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# THE EXCRETION OF ASCORBIC ACID AND DEHYDROASCORBIC ACID IN SWEAT AND URINE UNDER DIFFERENT ENVIRONMENTAL CONDITIONS\*

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A number of investigators (see (1)) have studied the occurrence of ascorbic acid in human sweat and several have reported its occurrence in considerable amounts. However, recent investigations have reported no ascorbic acid in sweat, but have reported the occurrence of dehydroascorbic acid. The data on the excretion of ascorbic and dehydroascorbic acid in sweat, with the exception of those contained in a later paper by Kirch, Cornbleet, and Bergeim (2), have been summarized by Sargent, Robinson, and Johnson (1).

Mickelsen and Keys (3) and Kirch *et al.* (2) have shown that rubber contains water-extractable materials which titrate as ascorbic acid in the 2,6-dichlorophenol indophenol titration method and thus have explained to some extent the high values reported in previous investigations in which sweat was collected by means of a rubber bag or rubber gloves.

In this paper we wish to present data on the occurrence of dehydroascorbic acid and of small amounts of free ascorbic acid in sweat, using the Roe and Kuether (4) 2,4-dinitrophenylhydrazine procedure. Urine samples as well as sweat were also analyzed by the 2,6-dichlorophenol indophenol titration method.

## EXPERIMENTAL

Four adult male subjects, aged 21 to 28 years, were maintained for 8 hours per day under controlled environmental conditions. During each 8 hour period the total skin excretion was collected, preserved with 3 per cent metaphosphoric acid or 0.5 per cent oxalic acid, and stored under refrigeration. No rubber equipment was used for the collection of the sweat.

When concentration data were desired, undiluted sweat was collected.

\* The data presented in this paper were taken from a project covered under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

*Ascorbic Acid and Dehydroascorbic Acid in Sweat*

Ascorbic acid analyses were carried out on samples of sweat and body washings by the 2,6-dichlorophenol indophenol titration method of Bessey (5). In all, 82 samples of human sweat collected during six 5 day periods were analyzed for ascorbic acid. The sweat collections were made either by running the lip of a beaker over the skin, or by washing the body after exposure to high temperatures for various periods of time. Out of these 82 analyses only nine showed positive titrations amounting to an excretion of 0.014 to 0.8  $\gamma$  of ascorbic acid per hour. Stability tests of ascorbic acid spread in dilute solution on the skin of the subjects, and removed at variable times later, gave recoveries of 75 to 90 per cent.

In view of the reports from other laboratories of the occurrence of ascorbic acid in sweat in rather high concentration, some explanation of our negative results was sought. It was found that, while sweat collected in glass was devoid of substances reducing 2,6-dichlorophenol indophenol, sweat collected by means of a rubber sleeve gave titrations with the dye equal in different collections to 0.59, 0.31, 0.44, and 0.76 mg. of ascorbic acid per 100 cc. of sweat. It was found that sweat collected under a cellophane covering, or under a cotton shirt impregnated with metaphosphoric acid gave no titration to 2,6-dichlorophenol indophenol. On the other hand, a 0.4 per cent aqueous solution of NaCl allowed to remain in contact with the rubber sleeve for 1 hour gave a titration with the dye corresponding to 0.43 and again to 0.18 mg. of ascorbic acid per 100 cc. Apparently, then, sweat collected in contact with vulcanized rubber leaches out something from the rubber, possibly a sulfhydryl compound, that reacts with 2,6-dichlorophenol indophenol. This has also been shown by Mickelsen and Keys (3) and by Kirch, Cornbleet, and Bergeim (2).

In all subsequent work on sweat the 2,6-dinitrophenylhydrazine procedure of Roe and Kuether (4) was used. It was found that dehydroascorbic acid does occur in sweat. Table I gives a summary of the average daily skin excretion of dehydroascorbic acid per hour for the four subjects on a constant low ascorbic acid diet under various environmental conditions. As would be expected, the loss of dehydroascorbic acid in the sweat increased under the more profuse sweating condition ("hot moist," Periods 18 and 19).

In Table II data on the concentration of dehydroascorbic acid in undiluted sweat are presented. These data were obtained in Period 24, during which profuse sweating was induced by a hot moist condition of 32° and a relative humidity of 65 per cent. During this entire week each subject received daily 500 mg. of ascorbic acid in two doses, 250 mg. after breakfast and 250 mg. after lunch. The average concentration was 66.5  $\gamma$  per 100 cc. of sweat.

Later in the course of the project it was decided to reexamine the occurrence of ascorbic acid in sweat by applying the more sensitive Roe and Kuether procedure for dehydroascorbic acid to the determination of free ascorbic acid. Undiluted sweat samples were obtained from four subjects after several weeks with no added ascorbic acid and after 1 week of 500

TABLE I  
*Average Hourly Skin Excretion of Dehydroascorbic Acid*

Period No	Environmental conditions		Average net loss in body weight	Dehydroascorbic acid*					Standard deviation†	Coefficient of variation†
	Dry bulb temperature	Relative humidity		Subject A	Subject B	Subject C	Subject D	Average		
	°C	per cent	gm	$\gamma$ per hr	$\gamma$ per hr	$\gamma$ per hr	$\gamma$ per hr	$\gamma$ per hr	$\gamma$ per hr.	per cent
17	28.3	42	67	48	30	19	18	29	43.5	150
18	36.9	67	443	97	74		106	92	66	72
19	37.2	66	760		153		120	136	101	74
20	43.3	25	652	20	90	26	94	56	46	82

\* Each value represents the average of five assays made on samples collected on the 5 consecutive days of the period.

† The standard deviation and coefficient of variation are for the individual samples, not for the averages for each subject given in the table.

TABLE II  
*Concentration of Dehydroascorbic Acid in Sweat\**

Subject	Dehydroascorbic acid per 100 cc. sweat during Period 24					
	Monday	Tuesday	Wednesday	Thursday	Friday	Average
	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$	$\gamma$
A	62	40	31	66	38	48
B	56	83	60	79	86	73
C	66	78	63	56	43	62
D	81	78	94	94	72	83
Average...						66.5†

\* The environmental conditions were 32° and 65 per cent relative humidity.

† Standard deviation, 17.9  $\gamma$ ; coefficient of variation, 27 per cent.

mg per day dosage. The Roe and Kuether method was applied to the sweat samples, first without the oxidation procedure for conversion of free ascorbic acid to dehydroascorbic acid, and then after the usual oxidation; the increase, if any, is due to ascorbic acid oxidized to dehydroascorbic acid. The results of these assays are presented in Table III; in which it can be seen that small amounts of free ascorbic acid were found in five

out of eight samples tested. A dosage of 500 mg. of ascorbic acid daily was associated with a marked reduction in the concentration of ascorbic acid in sweat and with a significant increase in the concentration of dehydroascorbic acid ( $P = 0.026$ ).

TABLE III

*Effect of Dosage on Concentration of Ascorbic Acid and Dehydroascorbic Acid in Sweat*

Period No.	Subject	Dosage*	Ascorbic acid	Dehydroascorbic acid	Total
		mg. per day	$\gamma$ per 100 cc.	$\gamma$ per 100 cc.	$\gamma$ per 100 cc.
40	C	0	29	25	54
	D	0	66	56	122
	E	0	15	31	46
	F	0	22	24	46
Average.....		0	33	34	67
41	C	500	0	159	159
	D	500	0	121	121
	E	500	0	102	102
	F	500	21	46	67
Average.....		500	5	107	112

\* Vitamin C given per day in addition to that in the diet, which was constant during both periods.

### *Urinary Ascorbic Acid Excretion*

24 hour collections of urine were made by the four subjects during certain experimental periods, and the urine samples were assayed for ascorbic acid by the 2,6-dichlorophenol indophenol titration method. The average excretion data of ascorbic acid in mg. per hour of each of the four subjects under varying levels of ascorbic acid intake and under different environmental temperatures and humidities are summarized in Table IV. These data show clearly the well known effect of dosage on urinary excretion of ascorbic acid. However, the positive effect of environment on urinary excretion of ascorbic acid was of particular interest in the present study. From the 14th through the 20th week the subjects were on a constant diet low in vitamin C. While on this diet, the imposition of hot moist conditions raised the ascorbic acid excretion approximately 2-fold, the values returning to their former level when the humidity was lowered even though the temperature remained high. This effect was unrelated either to water intake or to urinary volume. However, in two other periods (hot moist) in which there was a variable water intake from day to day (7.23 liters

Monday, Wednesday, and Friday and 2.83 liters Tuesday and Thursday during the 8 hour daily exposure period), a significant positive correlation between urine volume and ascorbic acid excretion of 0.47 was found.

TABLE IV  
*Urinary Excretion of Ascorbic Acid under Various Conditions of Intake and Environment*

Period No	Environmental conditions		Dosage* vitamin C	Wks on dosage	Length of collection period	Average ascorbic acid excretion in urine				
	Dry bulb temperature	Relative humidity				Subject A	Subject B	Subject C	Subject D	Average
	°C	per cent	mg. per day		days	mg. per hr.	mg. per hr.	mg. per hr.	mg. per hr.	mg. per hr.
1A	40.4	20	500	0	3	10.2	8.1	10.4	15.7	11.1
1B	41.1	19.5	0	0	2	6.8	3.4	4.6	4.1	4.7
7	40.4	45	500	5	5	41.6	36.3	26.7	44.0	37.2
14, 15	43.9	21.5	0	0	5	0.72	0.59	0.70	0.75	0.69
16, 17	28.1	42	0	0	5	0.51	0.55	0.33	0.48	0.47
18, 19	37.2	67	0	0	5	1.09	1.16		1.35	1.20
20	43.6	25.5	0	0	5	0.54	0.72	0.59	0.59	0.61

\* In addition to that in the diet

#### DISCUSSION

Under hot moist conditions (Periods 18 and 19, Table I) the average hourly loss of dehydroascorbic acid through the skin was 114  $\gamma$ . This amounts to only 2.74 mg. per 24 hours under conditions producing profuse sweating throughout the day. This value represents the total excretion of ascorbic acid and dehydroascorbic acid, since the Roe and Kuether assay procedure was used. Thus, it appears that under these conditions the skin loss would not noticeably affect the vitamin C requirement. While there is an average increase in the skin excretion of total vitamin C following dosage (Table III), this increase though highly indicative is not statistically significant ( $P = 0.09$ ). Another factor to be considered together with the skin loss of vitamin C under hot moist conditions is the increase in urinary loss, as is seen in Table IV, Periods 18 and 19. In these two periods the urinary loss averaged for all subjects 1.22 mg. per hour, or 29 mg. per day, as compared with a urinary loss of only 15 mg. per day during the two preceding "comfortable" periods (Nos. 16 and 17, Table IV). That is, on changing from comfortable to hot moist conditions there was an increased ascorbic acid loss of 14 mg. per day in the urine as compared with an increased loss of about 3 mg. through the skin. The most probable explanation of the low ascorbic acid concentrations found in sweat is a high threshold value for the excretion of ascorbic acid by the sweat glands.



## SUMMARY

1. Ascorbic acid and dehydroascorbic acid were determined in the sweat of four male subjects. The average concentration of ascorbic acid found in sweat was 19  $\gamma$  per 100 cc. (coefficient of variation, 109 per cent) and of dehydroascorbic acid 70.5  $\gamma$  per 100 cc. (coefficient of variation, 67 per cent) by the Roe and Kuether assay procedure.

2. The average total 24 hour excretion of dehydroascorbic acid varied from 0.8 mg. under "comfortable" conditions to 2.7 mg. under conditions inducing profuse sweating throughout the day.

3. The data indicate an increased skin excretion of dehydroascorbic acid following ascorbic acid dosage, and a decreased excretion of ascorbic acid.

4. The urinary excretion of ascorbic acid on a constant ascorbic acid intake was markedly increased under "hot moist" environmental conditions as compared to either "comfortable" or to "hot dry" conditions.

5. A positive correlation was found between urinary ascorbic acid excretion and urine volume when the water intake alternated from day to day between two widely different levels. However, on an approximately constant water intake no correlation was found between urine volume and urinary ascorbic acid excretion.

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# THE OCCURRENCE OF INOSITOL AND OF *p*-AMINOBENZOIC ACID IN SWEAT\*

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The occurrence of many of the water-soluble vitamins in sweat has been reported in recent years. Sargent, Robinson, and Johnson (1) have reviewed the literature on the occurrence of ascorbic acid, dehydroascorbic acid, thiamine, riboflavin, pantothenic acid, and nicotinic acid in sweat. We have demonstrated the presence of pyridoxine and its metabolites (2), choline (3), N<sup>1</sup>-methylnicotinamide (4), "folic acid" (5), and biotin<sup>1</sup> in sweat. In this paper we wish to report the occurrence of inositol and of *p*-aminobenzoic acid in sweat and the effect of a "hot moist" environment on the excretion of inositol.

## EXPERIMENTAL

Four adult male subjects, aged 21 to 28 years, were maintained for 8 hours per day, 5 days per week, under constant environmental conditions. The subjects were kept on a constant diet for 4 weeks. The effect of a change in environmental conditions from "comfortable" to hot moist on the dermal and renal excretion of inositol was studied during the last 2 weeks. During these two collection periods (Nos. 27 and 28) complete 8 hour skin excretion and 24 hour urinary collections were made. The samples were analyzed for inositol by a modification of the *Saccharomyces cerevisiae* G. M. method of Williams, Stout, Mitchell, and McMahan (6). In this modification, the yeast medium of Atkin, Schultz, Williams, and Frey (7) was used, the inositol being omitted and 1 mg. of pyridoxine being added per liter of double strength medium. The average hourly excretion of inositol in the urine and through the skin for the two experimental periods is given in Table I. From these data it is apparent that there was a greater skin loss of inositol during the hot moist period when sweating was profuse. The probability that this increase was brought about by a random combination of uncontrolled experimental factors is 0.0002. With a greater loss of inositol in the sweat under the hot moist condition, there appeared to be a compensatory decrease in the excretion

\* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Illinois.

<sup>1</sup> Unpublished data.

TABLE I

*Effect of Environmental Condition on Inositol Lost in Sweat and in Urine of Subjects on Constant Diet\**

Period No.	Environmental conditions		Subject	Net loss in body weight	Skin loss†	Urine loss‡	Total loss
	Dry bulb temperature	Relative humidity					
	°C.	per cent					
27	38.2	69.4	C	635	99	336	435
			D	863	151	366	517
			E	969	138	933	1071
			F	410	86	340	426
Average.....				719	118	494	612
28	29.2	52	C	27	26	367	393
			D	132	38	444	482
			E	80	20	1254	1274
			F	51	25	442	467
Average.....				72.5	27	627	654

\* Periods 27 and 28 are the 3rd and 4th weeks on this diet.

† 8 hour daily collection period.

‡ 24 hour daily collection period.

TABLE II

*Concentration of Inositol in Sweat*

Period No.	Dosage*	Subject	Inositol in sweat
22S	mg. per day		γ per 100 cc.
	50	C	19
	50	D	34
	50	E	15
Average.....	50	F	36
24S	0	C	22
	0	D	22
	0	E	12
	0	F	27
Average.....			21

\* Given in addition to that in the diet for 3 days preceding the test.

of inositol in the urine. Probably because of the wide variations observed, the decrease in urinary inositol during the hot moist conditions was not statistically significant ( $P = 0.13$ ).

In order to study the concentration of inositol in sweat, undiluted sweat samples were collected during 4 hour periods on Saturday mornings, during which a hot, humid environment was maintained. The effect of dosage of 50 mg. of inositol per day added to the normal diet on the concentration of inositol in sweat is shown in Table II. It was found that the increased intake of inositol does not significantly raise the concentration of inositol in the sweat ( $P = 0.2$ ).

TABLE III  
Concentration of *p*-Aminobenzoic Acid in Sweat

Period No.	Dosage*	Subject	<i>p</i> -Aminobenzoic acid in sweat	
			$\gamma$ per 100 cc.	$\gamma$ per hr.
23S	200	C	0.13	0.80
	200	D	1.70	14.73
	200	E	0.08	0.80
	200	F	0.24	1.17
Average..	200		0.54	
38S	0	C	0.20	0.24
	0	D	0.52	0.65
	0	E	0.23	0.49
	0	F	0.23	0.33
Average. .	0		0.295	
39S	100	C	0.29	1.13
	100	D	0.31	2.33
	100	E	0.21	1.37
	100	F	0.15	0.67
Average .	100		0.24	

\* In addition to that in the diet.

Another water-soluble factor which we have found in sweat is *p*-aminobenzoic acid. Undiluted sweat was collected following various dosages of *p*-aminobenzoic acid which were given for at least 3 days prior to the collection period. The sweat samples were analyzed for *p*-aminobenzoic acid by the *Acetobacter suboxydans* procedure of Landy *et al.* (8, 9). The concentrations of *p*-aminobenzoic acid found in sweat are given in Table III.

#### SUMMARY

1. Both inositol and *p*-aminobenzoic acid occur in human sweat.
2. The concentration of inositol in sweat averaged 21  $\gamma$  per 100 cc.
3. The concentration of *p*-aminobenzoic acid in sweat averaged 0.24  $\gamma$  per 100 cc.

4. The average excretion of inositol in sweat during the 8 hour daily exposure period was 0.118 mg. per hour under "hot moist" conditions. Under these same conditions the average urinary loss during 24 hour collection periods was 0.494 mg. per hour. The corresponding average losses under "comfortable" conditions were 0.027 mg. in sweat and 0.626 mg. in the urine.

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# FURTHER STUDIES OF THE PURIFICATION AND PROPERTIES OF THE AMYLASE OF *ASPERGILLUS ORYZAE*\*

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The mold, *Aspergillus oryzae*, is an important commercial source of amylase. In 1898, Takamine (1) described a method for the concentration of the amylase and called the product taka-diastase. This and similar products have found wide use in medicine and in industry. While amylase is the predominant enzyme of such preparations, other enzymes including maltase are also present and undoubtedly influence the products of the hydrolysis of starch.

The present report gives a brief summary of an extensive investigation of the purification and properties of this amylase. A procedure is described which yields preparations of exceedingly high amylase activity. These preparations give no evidence of maltase activity and have been used in detailed studies of the action of the amylase.<sup>1</sup>

## EXPERIMENTAL

Highly active concentrated dry products obtained on a commercial scale<sup>2</sup> from extracts of the mold were used as the starting material for this work. These were subjected to a number of procedures designed further to concentrate and purify the amylase. The success of each step was judged by the amylase activities attained. These were calculated to the common basis of amylase activity per mg. of total solids. All measurements of amylase activities were strictly comparable. For these, the amylase reacted for 30 minutes at 40° with 1 per cent soluble potato starch adjusted to 0.05 M sodium chloride and to 0.01 M acetate at pH 5.0 (2).

## Results

*Extraction*—Extracts of the amylase were made with a number of different solvents some of which had been recommended by other workers. The solvents included distilled water at room temperature and at 5°; 1, 5.

\* We wish to thank the Takamine Laboratory, Inc., for grants in aid of this investigation.

<sup>1</sup> Unpublished.

<sup>2</sup> We wish to thank the Takamine Laboratory, Inc., for the generous gift of this highly active material.

and 10 per cent sodium chloride; 50 per cent alcohol (3); and 0.05 M acetate buffer solutions adjusted to pH 3.6, 5.0, and 6.5. These pH values were selected because previous work in this laboratory suggested that the isoelectric point of the amylase might be at about pH 5.0. Aqueous solutions of papain in concentrations of 0.25, 0.50, 0.75, and 1.0 per cent at 5°, 25°, and 40° were also used, as several workers have reported increased success in the extraction of certain enzymes in the presence of this protease.

The results were expressed as amylase activity per mg. of total solids of the extracts. It was found that distilled water used at room temperature was as satisfactory as any of the other solvents studied for the extraction of the amylase from the alcohol-precipitated starting material used here.

*Fractionation with Ammonium Sulfate*—Previous work with this amylase (3) and with the amylases of barley and of malted barley (4, 5)<sup>3</sup> suggested the use of ammonium sulfate. This reagent was found to be very efficient for the fractionation of the amylase in the extracts and its use resulted in the most highly active products of this amylase so far obtained in this laboratory. Extracts with saccharogenic activities<sup>4</sup> of approximately 400 and amyloclastic activities<sup>5</sup> of approximately 2000 consistently yielded solutions with saccharogenic activities of approximately 3000 and amyloclastic activities of approximately 16,000. These preparations give no evidence of maltase activity.

Briefly, the method for obtaining these active preparations consists of fractional precipitation of aqueous extracts of commercial products with ammonium sulfate, suspension of the precipitates in a minimum of distilled water, dialyses to remove sulfate, concentration of the dialyzed solutions, measurement of amylase activities, and repetition of the fractionation and dialyses with the most active solutions until no further increases occur in the amylase activities per mg. of total solids of the dialyzed solutions.

Solid ammonium sulfate was used for the precipitation. The dialyses were carried out in nitrocellulose<sup>6</sup> bags which were tightly filled to prevent undue dilution of the solutions. Concentration of the dialyzed solutions

<sup>3</sup> Also unpublished work.

<sup>4</sup> The saccharogenic activity refers to the increase in the reducing value of the reaction mixture calculated as mg. of maltose per mg. of enzyme preparation when the hydrolysis of 1 per cent soluble potato starch is carried out for 30 minutes at 40° under certain specified conditions (2) and when the concentrations of the amylase are adjusted to give approximately the same (20 per cent) hydrolysis of the starch. The reducing values of the hydrolysis mixtures were determined iodometrically (6).

<sup>5</sup> The amyloclastic activity refers to the weight of starch hydrolyzed per unit weight of enzyme preparation to products which give a clear red color with iodine as judged by a modified Wohlgenuth method when the hydrolysis is carried out under certain specified conditions (2).

<sup>6</sup> Made from tubing purchased from the Visking Corporation, Chicago.

was accomplished by suspending the bags in the breeze of an electric fan (4). Suspensions were centrifuged at 5° in a mechanically cooled centrifuge.<sup>7</sup>

TABLE I

*Typical Data for Purification of Amylase of Aspergillus oryzae by Fractionation with Ammonium Sulfate with Interereng Dialyses*

Treatment with ammonium sulfate		Amylolytic activity (A)*	Saccharogenic activity (S)†	Ratio $\frac{(A)}{(S)}$
	gm. per 100 cc.			
Original extract		2,000	410	4.9
1st fractionation	0-30	9,300	1830	5.2
	30-35	8,100 (a)	1480 (a)	5.5
	35-40	5,600	1060	5.4
	40-50	2,400	1150	2.1‡
2nd " of 1st 0-30 gm. fraction		9,300	1830	5.2
	0-30	9,200 (b)	2030 (b)	4.5
	30-35	11,200 (c)	2320 (c)	4.8
	35-40	11,100 (d)	2210 (d)	5.0
	40-50	3,500	1060	3.3‡
3rd fractionation of (c) and (d)		11,200	2270	4.9
	0-30	10,200	1820	5.6
	30-35	8,900	1780	5.0
	35-40	11,200 (e)	2480 (e)	4.6
	40-50	15,600§	3310§	4.8

\* The amylolytic activity refers to the weight of starch hydrolyzed per unit weight of enzyme preparation to products which give a clear red color with iodine when the amylase acts on 1 per cent soluble potato starch for 30 minutes at 40° under certain specified conditions (2).

† The saccharogenic activity refers to the increase observed in the reducing value of the reaction mixture calculated as mg. of maltose per mg. of enzyme preparation when the amylase acts on 1 per cent soluble potato starch for 30 minutes at 40° under certain specified conditions (2) and when the concentrations of the amylase are adjusted to give approximately the same (20 per cent) hydrolysis of the starch. The reducing values of the hydrolysis mixtures were determined iodometrically (6).

‡ Fractions with these low ratios of amylolytic to saccharogenic activities were found to contain relatively high concentrations of maltase.

§ Further fractionation of these active solutions either with ammonium sulfate or with ethyl alcohol resulted in loss of activity.

Typical data for one series of fractionations are summarized in Table I.

In a similar manner, repeated refractionations of other fractions, designated a, b, and c, in Table I, also resulted in highly active solutions with saccharogenic activities of 2800 to 2900 and amylolytic activities of 13,000 to 15,000.

<sup>7</sup> Manufactured by the International Equipment Company, Boston.



Solutions of similar high activities were obtained from a number of starting materials of very different initial activities when the refractionations were continued in each case until constant maximum activities were reached. The number of refractionations necessary was found to depend upon the starting material but usually three to five refractionations were sufficient.

Attempts to purify the amylase by repeated fractionations with ammonium sulfate, but without the intervening dialyses, proved less satisfactory. The most active products thus obtained were about half as active as the best products resulting from the procedure described above.

Attempts to improve the purification of the amylase by adjusting the hydrogen ion activities of its solutions in the fractionation with ammonium sulfate or during the dialyses also failed to increase the activities of the final products or to change the ratios of the amylolytic activities.

Refractionation with ethyl alcohol of the highly active solutions of taka-amylase obtained by the fractionation with ammonium sulfate failed to increase their activities. This finding is in distinct contrast to the marked increases in activity obtained by alcohol fractionation of similarly purified solutions of the amylases of barley and of malted barley (4, 5).<sup>3</sup>

Other protein precipitants which have been suggested for the fractionation and purification of this or of other enzymes, namely sodium sulfate, magnesium sulfate, and basic lead acetate (7), were also investigated under a number of different conditions but gave less satisfactory results than the fractionation with ammonium sulfate.

A systematic study was made of the adsorption of the amylase by alumina gel. This procedure has been successfully used in the purification of a number of enzymes including pancreatic amylase (8, 9) and has been recommended as a step in the purification of the amylase of *Aspergillus oryzae* (7, 10). While the results of the adsorption study throw light on the properties of the amylase and are reported in detail elsewhere (11), they lead to the conclusion that adsorption by alumina gel is not an effective method for the purification of taka-amylase whether it is used alone or in conjunction with other procedures such as fractionation with ammonium sulfate, with ethyl alcohol, or with basic lead acetate (7).

The final highly active amylase preparations obtained by the fractionation with ammonium sulfate outlined above gave no evidence of maltase activity.

Partial inactivation of aqueous solutions of highly active taka-amylase at high temperatures (60° and 70°) or by acid (pH 3) or alkali (pH 9) caused no significant change in the ratio of their amylolytic to their saccharogenic activities. These results suggest the presence of a single amylase; they give no indication of the presence of two amylases of widely different labilities such as the  $\alpha$ - and  $\beta$ -amylases of malted barley (12).

An examination of the ratios of the amylolytic to the saccharogenic activities of the solutions obtained throughout the investigation also gives no evidence of the fractionation of two amylases, such as is observed in the fractionation by ammonium sulfate of the  $\alpha$ - and  $\beta$ -amylases of malted barley (4, 5).<sup>3</sup> Except for the fractions which contained relatively large concentrations of maltase, the fluctuations in the ratios of the two activities were relatively small. On the other hand, some of the fluctuations observed may have been due to the fractionation of small amounts of carbohydrases.

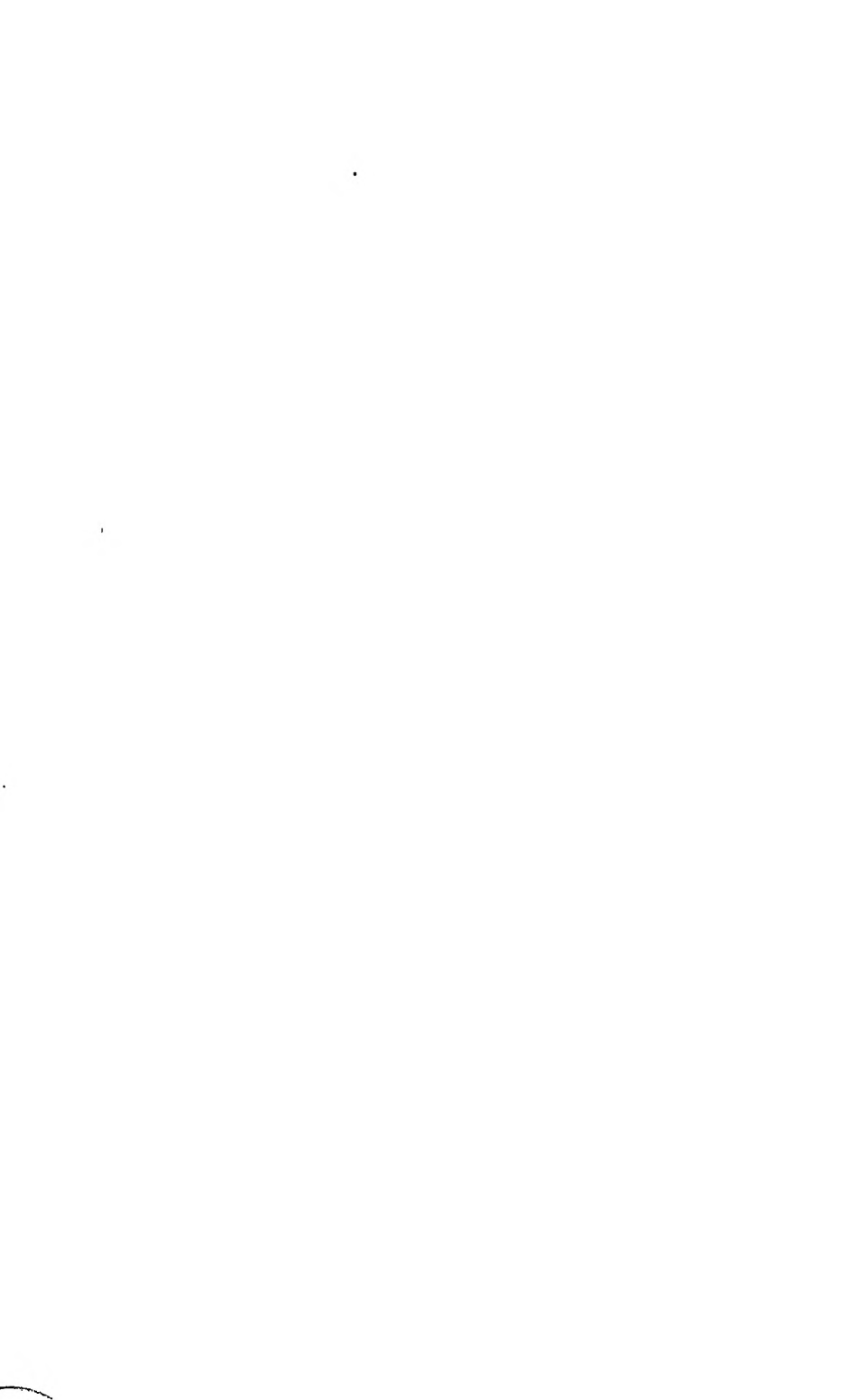
The highly active preparations of taka-amylase are being studied for their action upon a number of different substrates.

#### SUMMARY

An extensive study of the purification of the amylase of *Aspergillus oryzae* has been made. A procedure is described which yields preparations of exceedingly high amylase activities. These highly active products give no evidence of maltase activity and are being used in studies of the action and properties of the amylase.

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# THE ANTIOXYGENIC SYNERGISM OF TOCOPHEROL AND RICE BRAN EXTRACT IN THE PRESERVATION OF CAROTENE

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The occurrence of malignant hepatoma in rats ingesting a synthetic diet containing butter yellow may be prevented by the substitution of a high ratio of unsaturated fat in the diet. This protection against cancer could be neutralized by the additional inclusion of brown rice (1). These physiological reactions were explained by the demonstration *in vitro* that an oxidizing unsaturated fatty acid, such as linoleic acid, destroys butter yellow and that the oxidation is retarded by the presence of the brown rice. Aqueous extract of rice bran proved to be a potent antioxidant in preventing rancidity and the destruction of butter yellow when a linoleic acid solution of the dye was mixed into corn-starch (2). An attempt to demonstrate an *in vivo* action of the rice bran antioxidant in preserving carotene from oxidative destruction was unsuccessful; the simultaneous administration of carotene in linoleic acid with rice bran extract did not permit growth in vitamin A-deficient rats. Subsequent work (3) revealed that the remarkable stability afforded the linoleic acid-butter yellow-corn-starch mixture by rice bran was the result of a synergism between the rice bran extract and butter yellow; *i.e.*, the activity of the combination was much greater than that of either alone. With the recognition of synergistic factors in rice bran extract, the carotene stabilization experiments were revised. Rice bran extract was tested in combination with a preparation of mixed tocopherol, the latter being selected as typical of naturally occurring inhibitols and because it has been extensively studied for its protection of carotene while in the intestine from the destructive action of oxidizing fat (4-6). The experiments reported here reveal that the antioxygenic activity of tocopherol is synergistically enhanced by rice bran extract both *in vivo* and *in vitro*.

## Methods and Results

*In Vivo Stabilization of Carotene*—The effect of tocopherol and rice bran extract on the growth response of vitamin A-deficient rats receiving an oral supplement of carotene in linoleic acid was taken as a measure of the antioxidant action in the animal body.

21 day-old weanling rats from the laboratory colony were placed on the following vitamin A-deficient diet: vitamin-free casein (Smaco) 18, Primex 5, glucose 73, salt mixture 4 (U. S. P. XII). This diet differs from the standard U. S. P. diet in that it lacks yeast, the removal of yeast being necessary since yeast as well as other sources of the vitamin B complex (2) has been shown to contain factors that retard fat oxidation (7). The vitamin B requirements of the rats were furnished by a daily supplement of the crystalline vitamins, thiamine 20  $\gamma$ , riboflavin 25  $\gamma$ , pyridoxine 20  $\gamma$ , calcium pantothenate 100  $\gamma$ , and choline chloride 20 mg. Once weekly they received several drops of viosterol. Food and water were given *ad libitum*. On this dietary régime depletion of vitamin A reserves, as evidenced by cessation of growth and appearance of ocular symptoms, occurred between the 30th and 40th day. The rats were then divided by sex and weight into the number of groups desired. The supplement of crystalline carotene (Smaeo) and mixed tocopherol (Distillation Products, Inc.) dissolved in linoleic acid (Glyco Products, refined light) was prepared twice weekly and stored at 5°. The carotene content of the supplement was checked by spectrophotometric determination. The mixture of tocopherols was used as being representative of the naturally occurring form and because all three isomers possess approximately equal carotene-sparing action (6). Rice bran extract (Nopeo) was administered immediately after the oil supplement so that all the constituents concerned would be present at the same time in the rat intestine. All supplements were given orally by syringe. Survival time or increase in growth of the vitamin A-deficient rats was taken as a measure of physiological antioxidant activity of tocopherol and rice bran extract. The rats were weighed thrice weekly.

Harris, Kaley, and Hickman (6) have stressed the importance of a definite ratio of carotene to tocopherol for optimal protection. In all of the experiments reported here 0.2 mg. of mixed tocopherol was selected as a level which, while probably suboptimal for carotene sparing, would be more appropriate for the demonstration of a synergistic reaction with rice bran extract.

In the first preliminary experiment 5  $\gamma$  of carotene were given to all groups. The growth curves of Fig. 1 show that 5  $\gamma$  of carotene in 0.1 ml. of linoleic acid alone or with rice bran extract would not alleviate the steady decrease in weight of the vitamin A-depleted animals. The average survival time of Group 1 was 17.9 days; that of Group 2, receiving rice bran extract, was 12.8 days. The simultaneous administration of 0.2 mg. of mixed tocopherol with the carotene and linoleic acid resulted in a prompt positive growth response (Group 3). With the additional supplement of rice bran extract, still better growth was achieved (Group 4). The growth of this latter group approached that of the positive control, Group 5, in

which the rats received the vitamins in cottonseed oil solution. When the experiment was repeated with a larger number of animals, the same results were obtained; the group of rats receiving carotene and tocopherol in linoleic acid was approximately 10 gm. lighter after 7 weeks than was the group receiving the identical oil supplement plus rice bran extract. Statistical

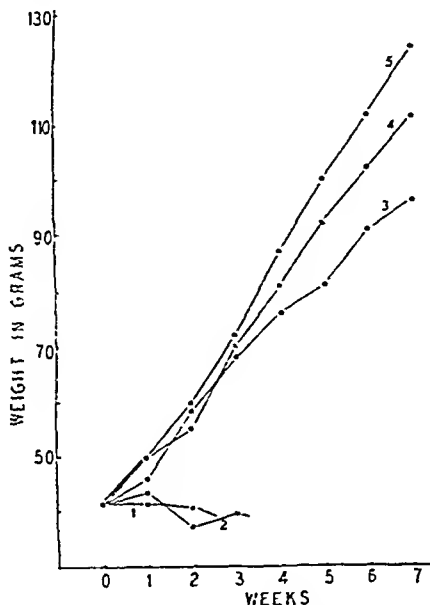


FIG. 1. Effect of mixed tocopherol and rice bran extract on the growth response of vitamin A-depleted rats receiving carotene in linoleic acid. The respective curves represent the results of groups treated as follows: Group 1, four males, three females, 5  $\gamma$  of carotene in 0.1 ml. of linoleic acid; Group 2, 2 males, 3 females, same as Group 1 + 0.2 ml. of rice bran extract; Group 3, 3 males, 2 females, 5  $\gamma$  of carotene + 0.2 mg. of mixed tocopherol in 0.1 ml. of linoleic acid; Group 4, 2 males, 3 females, same as Group 3 + 0.2 ml. of rice bran extract; Group 5, 3 males, 3 females, 5  $\gamma$  of carotene and 0.2 mg. of mixed tocopherol in 0.1 ml. of cottonseed oil.

analysis of the data (Table I) revealed that the difference of the mean weight increase of Groups 3 and 4 was definitely significant; the critical ratio<sup>1</sup> obtained was 3.9, and the probability of the difference in the mean being accidental was less than 0.01 per cent.

<sup>1</sup> Critical ratio =  $(M_1 - M_2) / \sqrt{(\sigma_1/\sqrt{N_1})^2 + (\sigma_2/\sqrt{N_2})^2}$ .  $M$  = mean,  $\sigma$  = standard deviation,  $N$  = the number of rats.

In an attempt to find conditions which would better demonstrate the synergism, additional groups of rats were supplemented with lower amounts of carotene. 2.0  $\gamma$  of carotene in 0.1 ml. of linoleic acid failed to maintain life even when protected with both tocopherol and rice bran extract. With a supplement of 3.5  $\gamma$  of carotene in 0.1 ml. of linoleic acid protected by the usual 0.2 mg. of mixed tocopherol, the growth rate of the rats at the end of 7 weeks was 8.5 gm. per rat per week, while a control group receiving an additional supplement of rice bran extract grew at a rate of 10.0 gm. per rat per week. This difference was also statistically significant.

The question arose as to whether this increased growth was the result of a greater intake of carotene due to the increased preservation of the ad-

TABLE I

*Effect of Mixed Tocopherol and Rice Bran Extract on Stabilization of Carotene in Linoleic Acid in Vivo*

Group No.	Daily supplement	No. of rats	Average survival time	Average weight gain at 7th wk.
			days	gm. per rat per wk.
1	5 $\gamma$ carotene in 0.1 ml. linoleic acid	9 $\sigma$ , 10 $\phi$	19.5	
2	Same + 0.2 ml. rice bran extract	9 $\sigma$ , 8 $\phi$	16.6	
3	5 $\gamma$ carotene + 0.2 mg. mixed tocopherol in 0.1 ml. linoleic acid	12 $\sigma$ , 16 $\phi$		9.2 $\pm$ 1.57*
4	Same + 0.2 ml. rice bran extract	10 $\sigma$ , 14 $\phi$		10.9 $\pm$ 1.46*
5	5 $\gamma$ carotene + 0.2 mg. mixed tocopherol in 0.1 ml. cottonseed oil	9 $\sigma$ , 12 $\phi$		11.8

\* Standard deviation.

ministered carotene by rice bran extract or to other nutritional factors present in the extract. Sullivan and Evans (8) have reported that the growth of carotene-supplemented rats was influenced by the amount of B vitamins ingested. As rice bran extract is certainly a good source of the vitamin B complex, an experiment was run in which one group of animals receiving 3.5  $\gamma$  of carotene and 0.2 mg. of mixed tocopherol in 0.1 ml. of linoleic acid was given, in addition to the usual supplement of the five necessary B vitamins, the following daily dose of other vitamin B factors: nicotinic acid 1 mg., inositol 1 mg., *p*-aminobenzoic acid 1 mg., and biotin 0.5  $\gamma$ . This group exhibited no better growth than a control group.

The results of the experiment presented in Fig. 2 offer evidence that the increased growth of rats receiving both tocopherol and rice bran extract

is the result only of increased carotene utilization and is not due to the presence of any other nutritional factors. After 7 weeks of supplementing, the rats of Group 1 receiving Supplement 1 (3.5  $\gamma$  of carotene and 0.2 mg. of mixed tocopherol in 0.1 ml. of linoleic acid daily) had reached an average weight of 131 gm.; Group 3 receiving the identical supplement plus 0.2 ml. of rice bran extract (Supplement 3) weighed 141 gm. It was reasoned that, if the difference in growth was the result of greater utilization of carotene alone, supplementing the rats of Group 1 with additional carotene should permit growth as good as that of Group 3. The eight rats receiving Supplement 1 (Fig. 2) were divided into two subgroups (Group 2). The

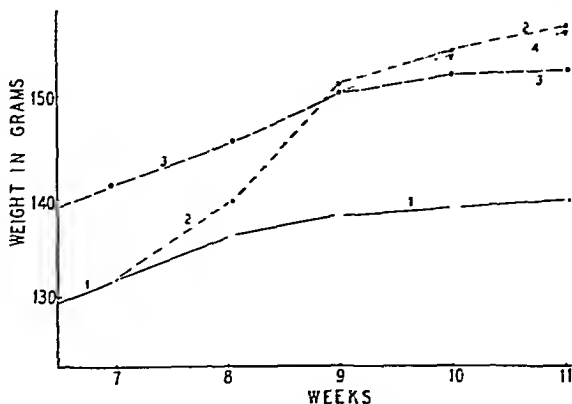


FIG. 2. *In vivo* stabilization of carotene in linoleic acid by mixed tocopherol and rice bran extract. The curve numbers represent the respective groups treated as follows: Group 1, 3.5  $\gamma$  of carotene and 0.2 mg. of mixed tocopherol in 0.1 ml. of linoleic acid; Group 2, same as Group 1 + separate supplement of 50  $\gamma$  of carotene in cottonseed oil; Group 3, same as Group 1 + 0.2 ml. of rice bran extract; Group 4, same as Group 3 + separate supplement of 50  $\gamma$  of carotene in cottonseed oil.

animals of Group 2 received a drop of carotene preparation (50  $\gamma$ ) daily with their vitamin B supplement, thus minimizing any possibility of destruction by the linoleic acid of the regular supplement which was administered later in the day. In 2 weeks time the average weight of the rats of Group 2 was equal to that of Group 3. Group 3 was then divided into two subgroups of four animals each (Group 4). Group 4 received the additional drop of carotene preparation (50  $\gamma$ ). Groups 2 and 4, receiving the excess of carotene, now showed identical growth curves, while Groups 1 and 3 continued their relative rate of growth. Each subgroup contained two males and two females. The fact that rice bran extract demonstrates a growth-promoting effect only on rats receiving a suboptimal amount



of carotene offers evidence of the existence of a factor or factors that are concerned only with carotene utilization. The postulation of an antioxidant synergist acting with tocopherol in retarding the destruction of carotene in the rat intestine by the oxidizing linoleic acid is borne out by the *in vitro* experiments described in the next section.

*In Vitro Stabilization of Carotene*—The effect of tocopherol and rice bran extract on the time required for the oxidative destruction of carotene in linoleic acid was taken as a measure of synergistic antioxidant activity. 1 ml. of a linoleic acid solution of carotene and tocopherol was pipetted into a number of 10 ml. Erlenmeyer flasks, carefully selected for uniformity of size. When rice bran extract was tested, 1 ml. of the extract was pipetted into the flask already containing the oil and the two solutions mixed by gently swirling the flask. The groups of flasks were then placed in the incubator at 30°; every day one flask from each group was removed and the carotene content determined. The contents of the flask were washed with approximately 10 ml. of petroleum ether into a separatory funnel, 20 ml. of alcoholic KOH were added to give a clear solution, 20 ml. of H<sub>2</sub>O were added, the mixture was shaken, and then, after separation into two layers, the water-alcohol solution was discarded. This procedure removed linoleic acid and oxidation products which interfered with the carotene determination. Carotene was determined in the proper dilution of the petroleum ether fraction by measurement of light transmission at 450, 460, 475, and 490 m $\mu$  with the Beckman spectrophotometer. The concentration of carotene was calculated by reference to standard curves. Rancidification of the linoleic acid was checked by determination of the peroxide number (9).

The results of a typical experiment testing the effect of tocopherol and rice bran extract on the preservation of carotene in linoleic acid are presented in Fig. 3. Under the conditions of the test, carotene in linoleic acid solution was completely destroyed in 24 hours (Curve 1); addition of 1 ml. of rice bran extract afforded no protection (Curve 2); 2 mg. of mixed tocopherol alone extended the time required for 50 per cent destruction of carotene to 2 days (Curve 3); while with both the tocopherol and 1 ml. of rice bran extract, approximately 60 per cent of the carotene was retained after 8 days (Curve 4).

*Linoleic Acid Stabilization by Tocopherol and Rice Bran Extract*—The determination of peroxide values on a similar set of samples correlates carotene destruction with oxidation of the linoleic acid. In samples containing only carotene in linoleic acid the peroxide number rises sharply on the 2nd day. The same is true when rice bran extract alone is present with the linoleic acid solution of carotene. In Fig. 4 are presented only the curves of the pertinent data demonstrating antioxidant synergism in

retarding linoleic acid oxidation and subsequent carotene preservation. With tocopherol present the end of the induction period, as characterized

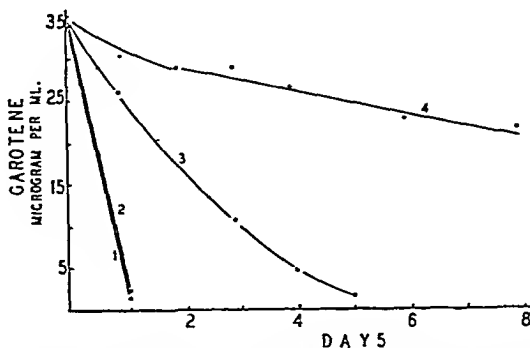


FIG. 3. Stabilization of carotene in linoleic acid by mixed tocopherol and rice bran extract. Curve 1, 35  $\gamma$  of carotene in 1 ml. of linoleic acid; Curve 2, same as for Curve 1 + 1 ml. of rice bran extract; Curve 3, 35  $\gamma$  of carotene and 2 mg. of mixed tocopherol in 1 ml. of linoleic acid; Curve 4, same as for Curve 3 + 1 ml. of rice bran extract.

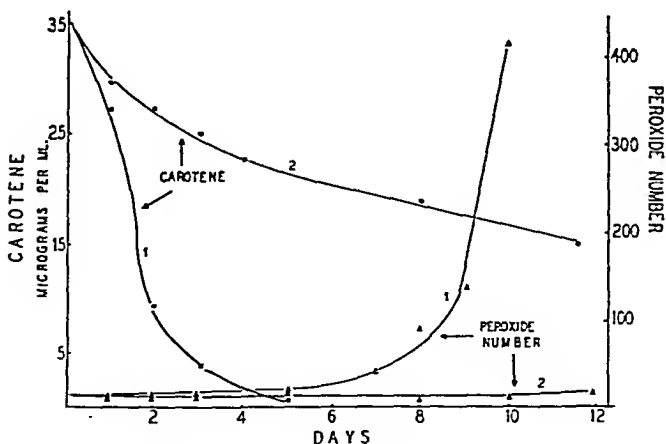


FIG. 4. Synergistic action of mixed tocopherol and rice bran extract in retardation of linoleic acid oxidation and subsequent preservation of carotene. Curves 1, 35  $\gamma$  of carotene + 2 mg. of mixed tocopherol in 1 ml. of linoleic acid; Curves 2, same as for Curves 1 + 1 ml. of rice bran extract.

by the sharp rise in peroxide content, occurs about the 8th day (Curve 1). With both tocopherol and rice bran extract present, relatively little oxida-

tion had taken place after 10 days; on the 15th day the peroxide number was still only up to 30 milliequivalents per gm. It is of interest to note in Fig. 4 that the sharp rise in peroxide number of Curve 1 occurs only after the complete destruction of carotene; this was found to be true in all samples, with or without antioxidants. Williams *et al.* (10) have reported a similar observation with carotene dissolved in mineral oil.

*Sparing of Tocopherol by Rice Bran Extract*—According to classical theory the end of the induction period of fat oxidation coincides with the complete destruction of antioxidants. Since rice bran extract is only effective in retarding oxidation when in combination with tocopherol, it would seem that factors in the extract preserve tocopherol from oxidative destruction or perhaps regenerate oxidized tocopherol in a manner similar to that described by Golumbic in his study of tocopherol and phosphoric acid synergism (11). Information on this point was sought by studying the effect of rice bran extract on the rate of destruction of mixed tocopherol in linoleic acid solution. Attempts to determine tocopherol by the Emmerie-Engel method (12) in the system described above were unsuccessful owing to the high concentration of interfering substances; namely, carotene and fatty acid oxidation products. Adsorption and solvent extraction studies yielded a low recovery of added tocopherol. For this reason the investigation was conducted on samples containing 100 mg. of mixed tocopherol per ml. of linoleic acid. By thus reducing the relative proportions of interfering substances and using the modified procedure suggested by Kaunitz and Beaver (13) for determination of tocopherol in the presence of fat, a satisfactory method was obtained. The test consisted of pipetting 5 ml. of the linoleic acid solution of tocopherol and carotene into two sets of 50 ml. Erlenmeyer flasks, 2 ml. of rice bran extract being added to one set. The flasks were maintained at 30° and at daily intervals 0.1 ml. aliquots were removed for tocopherol and carotene determinations. The results presented in Fig. 5 show a rapid destruction of carotene and tocopherol in the 1st few days; then the curves of Sample II, containing rice bran extract, level off and oxidation proceeds at a relatively slower rate (Curves 2). However, in Sample I with no rice bran extract, oxidation continues rapidly until complete destruction of tocopherol and carotene has occurred by the 24th day (Curves 1). At this time 40 per cent of the tocopherol still remains in Sample II. Rice bran extract in some manner preserves tocopherol from oxidative destruction. The carotene destruction curves of both samples follow very closely that of the tocopherol, confirming the ability of the rice bran extract to influence the state of oxidation existing in the fatty acid solution. It should be pointed out that while carotene is destroyed at the same rate as tocopherol the provitamin is not capable of antioxidant activity, at least not in the system under investigation.

Earlier work in this laboratory has revealed that the oxidation of linoleic acid with or without 2 mg. of tocopherol per ml., as measured by the peroxide number increase, is not affected by the presence of 35  $\gamma$  of carotene.

*Stabilization of Oils Pretreated with Rice Bran Extract*—In all of the preceding experiments linoleic acid rather than whole fat has been used. Since our interest has been with the physiological implications, linoleic acid was selected because unsaturated fat of the diet would be present in the intestine after enzymatic digestion, chiefly in the form of linoleic

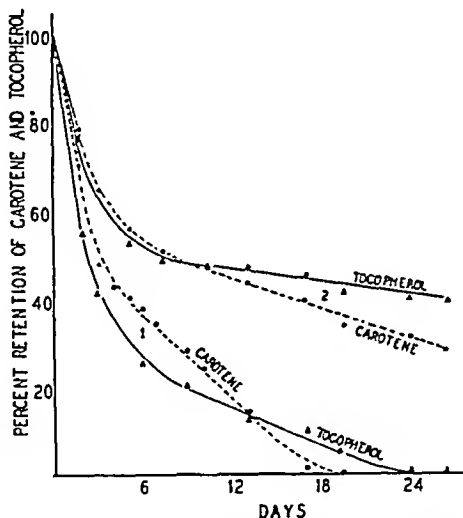


FIG. 5. Effect of rice bran extract on the preservation of mixed tocopherol and carotene in linoleic acid. Curves 1, Sample I, 500 mg. of mixed tocopherol and 3.5 mg. of carotene in 5 ml. of linoleic acid; Curves 2, Sample II, same as Sample I + 2 ml. of rice bran extract.

acid. Then too, linoleic acid, being more readily oxidizable, was a convenient substrate for fat oxidation studies. In the following experiments a study of the synergistic action of tocopherol and rice bran extract has been extended to cottonseed and soy bean oil. In these experiments a new technique was introduced. In previous experiments the rice bran extract remained in the assay flasks in contact with a linoleic acid solution of tocopherol and carotene during the oxidation. It was found that if the linoleic acid was first shaken thoroughly with the rice bran extract and then separated by centrifuging and filtering, the so treated linoleic acid made a much more stable solvent for the carotene and tocopherol.

In the experiment presented in Table II, 2 ml. of linoleic acid were shaken with varying amounts of rice bran extract. The mixture was separated and 350  $\gamma$  of carotene and 2 mg. of mixed tocopherol were dissolved in 1 ml. of the treated oil. The stability of the linoleic acid was determined by the rate of destruction of carotene. It is apparent from Table II that

TABLE II

*Preservation of Carotene by Tocopherol in Linoleic Acid Treated by Shaking with Varied Amounts of Rice Bran Extract*

Rice bran extract ml.	Per cent carotene retained at 30°					
	1 day	2 days	3 days	6 days	7 days	13 days
0.0	47.2	32.8	19.4	0	0	0
0.1	77.1	67.2	63.0	30.6	22.0	0
1.0	90.0	88.1	83.4	71.5	66.2	42.0
2.0	98.5	95.0	85.5	79.2	72.0	66.0
5.0	94.3	93.0	83.5	84.2	79.2	71.5
10.0	90.5	91.0	86.9	82.6	74.6	66.9

TABLE III

*Effect of Added Tocopherol and Rice Bran Extract on Preservation of Carotene in Cottonseed Oil*

Flask No.	Sample in 50 ml. Erlenmeyer flask	Per cent carotene retained at 30°			
		3 days	8 days	11 days	13 days
1	10 ml. cottonseed oil solution of carotene, 4.5 mg. per gm.	77.4	1.0		
2	Same + 5 ml. rice bran extract	88.5	20.7	1.0	
3	" as Flask 1 + mixed tocopherol, 4.5 mg. per gm.	93.1	34.4	9.4	2.1
4	Same + 5 ml. rice bran extract	90.4	59.7	49.0	43.1
5	10 ml. "treated"* cottonseed oil solution of carotene, 4.5 mg. per gm.	100.0	81.5	1.2	
6	Same + mixed tocopherol, 4.5 mg. per gm.	98.5	84.7	69.2	53.0

\* This oil contained 5 ml. of cottonseed oil that had been shaken with 5 ml. of rice bran extract.

increasing the amount of rice bran extract to 2.0 ml. results in increased protection of carotene. Above this level there seems to be no further increase in stabilization.

In Table III are presented data on the stabilization of cottonseed oil by tocopherol and rice bran extract. The most complete protection was found in Flasks 4 and 6, demonstrating the synergistic effect of tocopherol

and rice bran extract to be effective with cottonseed oil. Similar results were found with soy bean oil (Table IV). Stabilization of the oil by shaking with rice bran extract is accomplished to a greater degree with the cottonseed oil than with the soy bean oil (Flask 6, Tables III and IV). Addition of rice bran extract alone to either cottonseed or soy bean oil affords some protection (Flasks 2 and 5). This does not occur with linoleic acid, a fact which might be explained by the natural tocopherol already present in each of the oils.

TABLE IV

*Effect of Added Tocopherol and Rice Bran Extract on Preservation of Carotene in Soy Bean Oil*

Flask No.	Sample in 50 ml. Erlenmeyer flask	Per cent carotene retained at 30°		
		3 days	10 days	21 days
1	10 ml. soy bean oil solution of carotene, 4.5 mg. per gm.	70.5	1.1	
2	Same + 5 ml. rice bran extract	82.0	17.8	1.1
3	" as Flask 1 + mixed tocopherol, 4.5 mg. per gm.	76.2	9.4	
4	Same + 5 ml. rice bran extract	90.3	41.8	4.5
5	10 ml. "treated"* soy bean oil solution of carotene, 4.5 mg. per gm.	80.0	6.1	
6	Same + mixed tocopherol, 4.5 mg. per gm.	89.5	19.2	1

\* This oil contained 5 ml. of soy bean oil that had been shaken with an equal volume of rice bran extract.

## DISCUSSION

The rising interest in the participation of antioxidants in physiological fat reactions is amply evidenced by the number of recent publications. The majority of these investigations have been concerned with dietary factors which protect carotene, while in the intestine, from the destructive action of oxidizing fat. In the interpretation of results, it would be well to consider all such factors as members of one of the two groups tentatively suggested by Mattill (14) for classification of antioxidants; namely, (1) those phenolic compounds that are active *per se*, and (2) the synergists, acidic compounds, whose entire action is merely that of enhancing the effect of the phenolic antioxidants. Of the carotene-sparing factors, protective in the intestine, tocopherols (1-3), lauryl hydroquinone (3), and gossypol (15) may be considered as members of the first group. Examples of antioxidant synergists are those factors in soy bean phosphatide reported necessary for proper utilization of vitamins A and E by both the rat (16) and chick (17). Yeast also contains fat-soluble synergists (17),

a finding which cautions against the use of yeast as a source of the vitamin B complex for such experimental diets. Hickman *et al.* (18) have demonstrated a reinforcement of the antioxidant activity of mixed tocopherols by palmityl ascorbic acid as measured by carotene utilization and in the present paper a similar effect was demonstrated with rice bran extract. Since rice bran extract is effective only in combination with tocopherol, the antioxidant factor would appear to be of the acidic type; this, however, does not exclude the possibility of the presence as well of polyphenolic compounds which may be antioxygenic in themselves but whose activity is not demonstrable under the conditions of the experiment. Such combination of antioxidants appears to play a rôle in the antioxygenic reactions involved in the production of malignant hepatoma in rats by feeding N, N-dimethylaminoazobenzene (butter yellow) (1). All the examples cited here concern retardation of fat oxidation in the intestine; our knowledge of the participation of antioxidants in tissue reactions rests with the observation of Houchin and Mattill (19) that tocopherol phosphate lowers the high oxygen consumption of dystrophic rabbit muscle. In the complex biological oxidations of the animal body, it would seem more than likely that antioxidation would be the result of a multiple synergy rather than that of a single entity. This would seem especially true when one considers that of the large number of acidic compounds, both organic and inorganic, shown by Olcott (20) to be antioxidant synergists, a good proportion are constituents normally present in tissue.

#### SUMMARY

1. The growth rate of vitamin A-deficient rats receiving an oral supplement of carotene and tocopherol in linoleic acid solution is increased by the additional supplement of rice bran extract.

2. This growth-promoting effect is only evident with rats on a sub-optimal carotene intake, indicating the presence of factors in rice bran extract that permit a greater utilization of carotene.

3. *In vitro* experiments reveal that rice bran extract acts synergistically with mixed tocopherols in retarding oxidation of linoleic acid and consequently preserves carotene.

4. Rice bran extract is effective in that it delays the destruction of tocopherol in linoleic acid solution.

5. The antioxidant synergism of rice bran extract and tocopherol is also effective with cottonseed and soy bean oil.

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# CYANIDE LOSS FROM MEDIA IN STUDIES OF TISSUE METABOLISM IN VITRO\*

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In the course of experiments in this laboratory on the general subject of oxygen poisoning it became necessary to study the metabolism or function of tissues *in vitro* in the presence of cyanide. Although cyanide is frequently used as an inhibiting agent, a methodical experimental investigation of the factors influencing cyanide concentration under the conditions of the Warburg and similar techniques is apparently lacking. To measure the oxygen consumption of tissues in a closed respirometer it is necessary to include an alkali inset to absorb  $\text{CO}_2$ . Under these circumstances, cyanide present in the medium distills over to the alkali. To prevent this, the inclusion of cyanide in the alkali inset was advocated by Walker (1), and later Krebs (2). Neither author, however, gave more than empirical data for the assembly of experimental systems. Van Heyningen (3) showed the importance of the initial concentration of cyanide in the alkali, although he was chiefly concerned with other influences upon cyanide inhibition. He found that, at an initial cyanide concentration in the medium of  $10^{-3}$  M, the respiration of rat kidney slices was 80 per cent inhibited provided cyanide was also present initially in the  $\text{CO}_2$ -absorbing alkali inset, but only 8 per cent when cyanide was omitted from the alkali.

The first theoretical analysis of the cyanide equilibrium between a neutral medium and an alkali inset was given by Umbreit (4), who calculated the cyanide required in the alkali to prevent loss from various initial concentrations in the medium. In Umbreit's table, however, the total amount of alkali to be added to the inset ranged from 0.2 to 20 microequivalents. These amounts appear too small for most studies by the Warburg method. No experimental data are included in Umbreit's discussion.

The present paper develops a further theoretical approach, and compares experimental with calculated equilibria. The results offer a practical basis for the assembly of experimental systems in which cyanide is used.

\*The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

*Theoretical*—Under otherwise constant conditions it may be assumed that the rate of distillation of cyanide from the medium to the alkali inset or vice versa will depend upon the concentration of hydrocyanic acid. Accordingly, equilibrium will be established when the respective concentrations of HCN are equal. Thus,

$$(\text{HCN}) = \frac{(\text{H}^+)'}{(\text{H}^+) + K'} T' = \frac{(\text{H}^+)''}{(\text{H}^+)'' + K''} T'' \quad (1)$$

where  $(\text{H}^+)'$  and  $(\text{H}^+)''$  are hydrogen ion concentrations in the alkali and medium respectively,  $K'$  and  $K''$  are apparent acid dissociation constants of HCN in the alkali and medium respectively, and  $T'$  and  $T''$  are total cyanide concentrations in the alkali and medium respectively.

In a dilute cyanide solution (0.010 M), no other salt being present, the author determined electrometrically  $K''$  to be  $10^{-9.2}$ , a value in accord with that given in the literature (5). Further, since the medium, in most *in vitro* work is approximately  $(\text{H}^+)'' = >10^{-7}$  and the alkali insets are at  $(\text{H}^+) = <10^{-12}$ , it is at once obvious that  $(\text{H}^+) < K'$  and  $(\text{H}^+) > K''$ . Equation 1 then becomes, approximately,

$$\frac{T'}{T''} = \frac{K'}{(\text{H}^+)} \quad (2)$$

It is significant to note that the dissociation constant which remains in the equation is that of the HCN in the alkali, not that in the medium. But the assumption usually made in applying expressions similar to Equation 1 to the assembly of experimental systems is that  $K' = K''$ . As a hypothetical example with this figure for both the medium and alkali, when the alkali is 0.1 M, i.e.  $\text{pH}' = 13$  (a concentration commonly used),  $T'/T'' = 6300$ . In other words, the KCN in the alkali would have to be 6 M in order to give a constant concentration in the medium of 0.001 M. Furthermore since 6 M KCN in alkali is a saturated solution, 0.001 M KCN in the medium would be the maximum possible concentration which could be maintained constant.

Tables hitherto published for practical use, for example those by Umbreit (4), have been developed on the same assumption. To maintain the concentration of KCN in the medium at a value greater than 0.001 M the theory requires that the pH in the alkali inset be diminished; i.e., that the concentration of the alkali be lowered. But this leads to the further difficulty that the total amount of alkali becomes too small for practical purposes unless the capacity of the inset is made impractically large.

It soon became apparent, however, that the predictions derived as above were so much at variance with observations that important factors were

being neglected. Two are discussed in this paper. (a) As is well known, acid dissociation constants are markedly influenced by the ionic environment; hence the theory must include differences in  $K$  between solutions of widely different salt concentration. This necessitated measurement of the acid dissociation constant of HCN under the actual conditions used in respiratory studies by the Warburg method. (b) Experimental assemblies present other variables not practically included in any theoretical analysis, making individual controls imperative in every case.

### Methods

Recrystallized KCN was made up as stock in 0.001 M NaOH solution and the concentration checked at intervals by gravimetric analyses as AgCN. Other cyanide determinations were by the colorimetric method of Smith (6), in which the addition of alkaline picrate to cyanide produces a reddish brown color due to the formation of isopurpurate. The method was adapted for use with the Klett-Summerson photoelectric colorimeter, with Filter 54. Measurements were made from 0.1 to 1.0 micromole with a reproducibility of  $\pm 2$  per cent and it was found that multiple determinations could be run simultaneously in 15 or 20 minutes.

As pointed out by Smith (6), glucose and other substances present in a glucose-phosphate-Ringer's medium interfere with the picrate method for cyanide. Accordingly, a simple 0.040 M phosphate buffer at pH 7 was used as the medium.

### EXPERIMENTAL

*Determination of Value of Dissociation Constant of HCN in Concentrated Solutions of Cyanide in Alkali*—This was done by measuring the conditions necessary for equilibrium between the cyanide in the alkali and that in the medium. Since the medium is at pH 7.0 and the observed value of  $K'' = 10^{-9.2}$  (see above), the cyanide in the medium is practically all in the form of HCN. This assumption allows of the calculation of  $K'$  in the alkali inset. 2.0 ml. portions of weakly alkaline KCN were placed in the main compartments of standard Warburg respirometers, 1.0 ml. of phosphate buffer in the side sacs (sufficient to give a final concentration after mixing of 0.04 M, and pH 7.0), and 0.5 ml. of 0.1 M NaOH in the center wells. KCN was initially present in the NaOH inset, as shown in the results. The vessels were attached to manometers and equilibrated at 38° in a water bath. The contents of the side sacs and main compartments were then admixed and the time taken. The vessels were shaken thereafter at 5 cm. amplitude and 100 oscillations per minute. The vessels were taken down at various times, alkali was added to the main compartment to prevent loss of the remaining cyanide, and the contents of the

center well were removed by suction. The vessels were kept stoppered for the later determination of cyanide by the picrate method. In all cases the initial cyanide was determined at the time of tipping, and the concentration of the stock was checked at intervals by the gravimetric method, since cyanide is slowly lost even from alkaline solution.

Fig. 1 shows the effects of varying the initial concentration of cyanide in the alkali inset upon the concentration of cyanide in the medium after a standard 30 minute period of equilibration. The initial cyanide concentration was 0.0027 M. By interpolation, it is seen that there was no gain or loss from the medium during equilibration with approximately 5 M KCN in the alkali. In other words at this point the concentration of HCN in the medium and alkali inset is the same; viz., 0.0027 M. When Equation 1 is applied to the conditions in the alkali inset,  $K' = 10^{-9.72}$ , a significantly different value from that obtained for dilute solutions ( $K''$

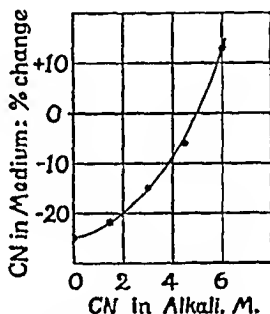


FIG. 1. The per cent gain or loss of cyanide in a neutral medium in 30 minutes at 38°, in the presence of a 0.1 M NaOH inset with varying initial concentrations of cyanide. The initial concentration of cyanide in the medium was 0.0027 M in all cases.

$= 10^{-9.20}$ ). When  $T'/T''$  in this case was found to be 1900, the value predicted by assuming  $K' = 10^{-9.2}$  was shown above to be 6300.

It soon became apparent, however, that this new determination of  $K'$  in concentrated cyanide solution did not clear up all of the difficulties, for when the initial concentration of cyanide in the medium was greater than 0.003 M the theoretical value of  $T'/T'' = 1900$  at equilibrium was not even remotely approached. These experiments are given in the following two sections.

*Cyanide Concentration in Medium As Function of Time, with No Cyanide Initially Present in Alkali*—The procedure was essentially the same as in the preceding experiment. Fig. 2 shows the results with two different concentrations of cyanide initially in the medium. When the initial cyanide concentration was 0.030 M, there was a rapid distillation of cyanide from the medium to the alkali (50 per cent) in the first 5 minutes. When

the initial concentration was much lower, *i.e.* 0.008 M, the loss from the medium was slow (25 per cent in 45 minutes).

In the case of the upper curve of Fig. 2, the final cyanide determination was made at apparently complete equilibrium, and shows a loss of 0.002 M concentration, or 60 micromoles total. Assuming the cyanide in the gas phase to be negligible, which is consistent with its high solubility, this means a final concentration of cyanide in the alkali of 0.12 M. In this case, then, the ratio  $T'/T'' = 12$ . In other words, the result is far removed from that predicted by theory, *viz.* 1900, indicating the presence of factors not included in Equation 1.

A possible explanation for this result lies in the physical characteristics of the system. The standard Warburg vessels used were equipped with

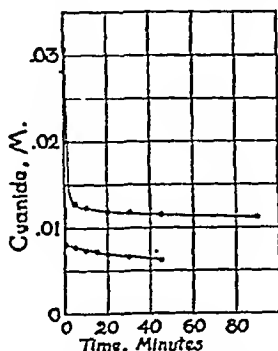


FIG. 2. The cyanide concentration in a neutral medium as a function of time, in the presence of a 0.1 M NaOH inset with constant shaking at 33°, no cyanide being initially present in the alkali.

center wells of approximately 6 mm. internal diameter, and height 25 mm. It was observed that the 0.5 ml. contained in these center wells was agitated to a negligible degree by the shaking. For this reason admixture of absorbed cyanide throughout the entire volume of the alkali in the inset was almost entirely dependent on simple diffusion. A high concentration of cyanide could thus be rapidly reached at the surface of the alkali. Since cyanide was absorbed from the gas phase as free HCN, there may also have been a significant lowering of pH locally, with a consequent increase in the proportion of cyanide as free acid. A close approach to an equilibrium was thus established with a much higher concentration in the medium than that predicted by theory.

In Fig. 2, the lower initial concentration of cyanide in the medium probably resulted in a less marked local effect at the surface of the alkali, and a consequently more steadily continued approach to true equilibrium.

*Cyanide Equilibrium As Function of Initial Concentration in Medium, with Standard Concentration in Alkali*—Fig. 3 compares the experimentally determined equilibria with those predicted by Equation 2, in which  $T'/T'' = 1900$ . The theoretical curve is seen to be almost exactly horizontal at the 0.003 M level. This follows from the fact that any distillation from the medium will cause a change in cyanide concentration in the alkali which is negligible with respect to its initial concentration of 5 M. In other words, within obvious limits, distillation should proceed from the medium to the alkali until the cyanide concentration in the medium reaches 0.003 M, regardless of its initial concentration. Instead of this, the result shows that distillation has proceeded only until a constant proportion of

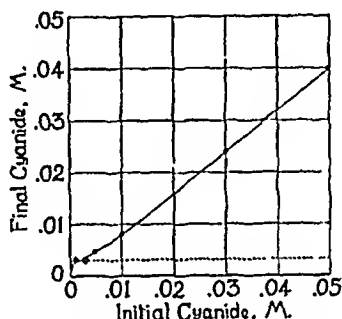


FIG. 3. Equilibrium (final) concentration of cyanide as a function of its initial concentration, following 90 minutes continuous shaking at 38°, in the presence of 5 M KCN in 0.1 M NaOH (80 per cent saturated). The circle represents the point of zero distillation shown in Fig. 2. The broken line is the function predicted by theory.

the initial cyanide has been lost to the alkali (20 per cent). This result could be explained in a manner similar to that of the previous experiment.

*Conclusions*—While it has been shown that previous theoretical analyses of cyanide equilibria between two adjacent solutions of differing pH within a closed system have been based upon erroneous assumptions, the experiments presented demonstrate that no simple theoretical analysis can be depended upon for the design of experimental assemblies for techniques of the Warburg type. It is therefore necessary in all cases to control experimental assemblies by methods similar to these given. Furthermore, a system of the type studied is capable of maintaining a cyanide concentration in the medium up to any value reasonably desired, without the reduction in alkali concentration or increase in alkali volume previously thought necessary.

In addition, it must be said that while previous workers have often made a sufficient approximation to the conditions necessary for the maintenance

of known levels of cyanide concentrations in Warburg studies, they may in some cases have had concentrations sufficiently above or below the assumed to place discussion of the relation between cyanide concentration and the degree of respiratory inhibition upon a dubious basis.

#### SUMMARY

1. Theoretical analyses of factors governing cyanide loss from neutral media to alkali insets within respirometers of the Warburg type are shown to demand inclusion of separate dissociation constants for HCN in dilute and concentrated solutions.

2. Experiments are given to show the inadequacy of any simple theoretical analysis to predict the conditions required in experimental assemblies to prevent alteration of the cyanide concentration in the media.

3. The necessity is emphasized for the control determination of cyanide in all cases in which its concentration must be known accurately under conditions of the Warburg technique.

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# THE ISOLATION OF PREPARATIONS OF THROMBOPLASTIC PROTEIN FROM HUMAN ORGANS\*

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Publications from this laboratory have, during the past few years, drawn attention to the isolation from beef lungs of an extremely potent activator of blood clotting, the thromboplastic protein, and to the chemical and physical properties of this substance. This work was summarized a short time ago (1). It has recently been shown that practically all the thromboplastic activity of the tissue is localized in this presumably cytoplasmic fraction which is a lipoprotein, containing pentose nucleic acid, of a very high particle weight; the other tissue proteins and the cell nuclei are devoid of activity (2). The properties of similar fractions from other organs and other animal species remain, however, to be determined.

Because of their possible application in the treatment of hemophilia and related diseases, an orienting study of highly purified preparations of the thromboplastic protein of human origin appeared of interest. The organs examined were the placenta and, for purposes of comparison with previous work, the lungs. The methods of isolation and assay followed the procedures presented previously (2-4).

The strong thromboplastic activity of placenta extracts has long been known (5, 6). Attempts at the therapeutic application of such preparations are of more recent date (7, 8). Normal human lungs appear to have been rarely investigated, although a thromboplastically active extract has been obtained from the lungs of a hemophiliac (9).

The preparations from placenta and human lungs were, like the thromboplastic protein of beef lungs, electrophoretically homogeneous lipoproteins of very high particle weight. Their thromboplastic activity was considerable, although not quite as impressive as that of the comparable preparations from beef lungs. The activity was confined to one tissue fraction; the soluble tissue proteins and the coarse tissue particles were, in confirmation of previous findings (2), practically inactive.

All of the thromboplastic protein preparations examined in detail, namely the substances isolated from human and beef lungs and from

\*This work has been supported by a grant from the John and Mary R. Markle Foundation. This is Paper XX of a series of studies on the chemistry of blood coagulation.

placenta, have one property in common: they are lipoproteins of a very high particle weight. Apart from this, however, very little can be said definitely with respect to their similarities and dissimilarities. The preparation from placenta appears to be less stable than the lung preparations and has a lower electrophoretic mobility. It is not yet possible to state whether one is dealing here with a family of conjugated proteins having the same active grouping in common; the nature and the function of such a grouping would be purely conjectural.

#### EXPERIMENTAL

*Human Placenta*—The fresh tissue<sup>1</sup> was cleaned, stripped of the membrane, cut into small pieces, and washed with cold water. The material (618 gm.) was gradually added to a mixture of 350 cc. of 0.29 M sodium chloride solution and 350 gm. of crushed ice that rotated in a high speed mixer. The supernatant resulting from the centrifugation of the mixture at 4000 R.P.M. (1900 *g*) for 30 minutes was fractionated by the methods described previously (2-4). Data on the various fractions will be found in Table I. The coarse lipoprotein aggregates (Fraction P-1), sedimented at 5000 *g* for 30 minutes, consisted of almost white voluminous flakes; the thromboplastic protein (Fraction P-2), sedimented at 31,000 *g* for 120 minutes, was a white light felt.<sup>2</sup> The soluble tissue proteins (Fraction P-3), isolated as described recently (2), formed dark pink flakes.<sup>3</sup> The reaction for acetal phosphatides was positive in Fractions P-1 and P-2. All fractions appeared to be free from desoxypentose nucleic acid and gave no metachromatic reaction with toluidine blue.

*Human Lungs*—The extraction of the tissue (630 gm.)<sup>4</sup> and the fractionation of the extract were carried out in the usual manner. The properties of the compounds are summarized in Table I which also contains data on similar fractions from beef lungs. Fraction L-1 consisted of a black powder,<sup>5</sup> Fraction L-2 of a light gray felt, Fraction L-3 of pink

<sup>1</sup> We are greatly indebted to Dr. S. Graff for this material.

<sup>2</sup> This preparation was not monodisperse in the ultracentrifuge. The main component had a sedimentation constant  $s_{20} = 270$  Svedberg units (in borate buffer of pH 8.4). We are very grateful to Dr. D. H. Moore for this determination, as well as for the performance of the electrophoresis experiments.

<sup>3</sup> A small protein portion (comparable to Fraction 3a in Table II (2)), 0.22 gm. per kilo of tissue, separated during the dialysis. It contained N 13.3, P 0.2 per cent and had no thromboplastic activity.

<sup>4</sup> We wish to thank Dr. E. E. Sproul for this specimen.

<sup>5</sup> It is well known that the lungs of adults, especially of city dwellers, are contaminated with inhaled carbon particles. The fact that all sedimentable protein fractions from human lungs, including the thromboplastic protein, were gray or black indicates the presence in lungs of carbon particles of a variety of sizes, even down to the submicroscopic dimensions of the tissue particles (of a diameter of 100  $m\mu$  and less).

flakes. The results of the various color reactions were identical with those obtained with the comparable fractions from placenta.

*Extraction of Lipids*—The thromboplastic protein preparations were suspended in a mixture of equal parts of absolute alcohol and peroxide-free

TABLE I  
*Thromboplastic Protein Preparations*

Source	Frac- tion No.	Designation	Yield per kilo- tissue	N	P	Electrophoresis*		
						Buffer	pH	Mobility (descend- ing boundaries)
			grs.	per cent	per cent			$\mu \times 10^5$
Human pla- centa	P-1	Coarse lipo- protein aggregates	0.74	9.2	1.1			
	P-2	Thrombo- plastic protein	1.08	8.7	1.4	Borate	8.2	-5.8
	P-3	Soluble proteins	19.4	14.7	0.2	Phos- phate	7.4	-1.1, -3.2, -3.7, -5.2, -6.7
Human lungs	L-1	Coarse lipo- protein aggregates	0.80	6.8	0.9			
	L-2	Thrombo- plastic protein	0.95	6.5	1.3	Borate	8.2	-7.1
	L-3	Soluble proteins	22.8	13.9	0	Phos- phate	7.4	-0.8, -2.5, -3.2, -4.2, -5.4
Beef lungs	1	Coarse lipo- protein aggregates	0.73	7.0	2.0			
	2	Thrombo- plastic protein	0.63†	7.7†	1.5†	Borate	8.5	-8.3
	3b	Soluble proteins	10.3	15.0	0.2	"	8.4	-1.4, -3.7, -5.3, -9.0

\* In preparations in which more than one component was observed, the main components are in bold-faced type.

† These figures represent the average of ten individual preparations. The yields are subject to considerable fluctuation.

ether (25 cc. of the solvent mixture per 100 mg. of substance) and heated under a reflux for 24 hours in an atmosphere of nitrogen. The extraction residues were collected by centrifugation, washed repeatedly with alcohol-ether (1:1) and with ether, and dried to constant weight *in vacuo* over

TABLE II  
*Extraction of Lipids from Thromboplastic Protein Preparations*

	Human placenta (Fraction P-2)					Human lungs (Fraction L-2)					Beef lungs (Chargaff, Bendich, and Cohen (4))			
	Weight	Per cent of total	N	P	Iodine value	Weight	Per cent of total	N	P	Iodine value	Per cent of total	N	P	Iodine value
	mg.		per cent	per cent		mg.		per cent	per cent			per cent	per cent	
Total, subjected to extraction....	199.4	100	8.7	1.4		101.4	100	6.5	1.3		100	7.6	1.6	
Protein residue.....	113.0	56.7	14.2	0.34		43.0	42.4	12.3	0.64		47.5	13.4	0.38	
Lipids (crude).....	79.2	39.7				54.0	53.3							
" (purified).....	72.0	36.1	1.5	2.3	74.0	47.6	47.0	0.95	1.5	63.8	38.8	1.32	5.6	50.6

$P_2O_5$ . The crude lipids, recovered by evaporation and drying *in vacuo* of the supernatants and washings, were dissolved in ether and the turbid solutions were twice shaken with a 10 per cent sodium chloride solution. The aqueous layers were washed with ether in the centrifuge. The combined ethereal solutions were dried over anhydrous sodium sulfate and evaporated *in vacuo*. Solutions of the residues in chloroform were again clarified with  $Na_2SO_4$  and the purified lipids were recovered from the filtrates.

The extraction experiments are summarized in Table II. For comparison, results obtained with the thromboplastic protein from beef lungs (4) are included.

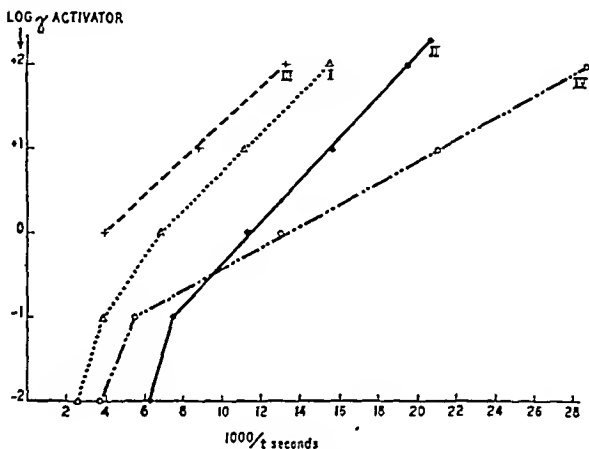


FIG. 1. Coagulation of normal human plasma (obtained by high speed centrifugation) by thromboplastic protein preparations of human origin. Curve I, Fraction I-2 from lungs; Curve II, Fraction P-2 from placenta; Curve III, Fraction P-2 from placenta after 5 weeks storage; Curve IV, thromboplastic protein from beef lungs (2). In the expression  $1000/t$ , plotted as the abscissa,  $t$  corresponds to the clotting time in seconds.

**Thromboplastic Activity**—The assays were carried out by the method recently described on normal human plasma (obtained by high speed centrifugation) as the substrate (2). The activities of the thromboplastic protein preparations from human lungs and placenta are represented in Fig. 1. It will be observed that the placenta preparation (Fraction P-2), stored in the dry state in the refrigerator for 5 weeks, showed a considerable diminution of activity, in contrast to the thromboplastic protein from beef lungs which was used as the standard. It may be mentioned that the straight lines representing the activities of the preparations from human

organs (Curves I, II, III, Fig. 1) differed in slope from those obtained with the beef lung fractions (Curve IV). Whether this is significant cannot be said.

Of the other substances examined Fraction P-1 had a slight activity; Fractions P-3, L-1, and L-3 were completely inactive.<sup>6</sup>

The author is very grateful to Miss Helen Fabricant for technical assistance.

#### SUMMARY

Highly active thromboplastic protein fractions were isolated from human lungs and placenta. Some of the properties of these substances, which are lipoproteins of a very high particle weight, are discussed. Other tissue fractions examined were devoid of thromboplastic activity.

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<sup>6</sup> Fraction L-3, when tested previous to dialysis, was very slightly active.

## STUDIES ON THE LIBERATION OF COMPOUNDS IN THE FOLIC ACID GROUP\*

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The apparent "folic acid"<sup>1</sup> activity of a food material is dependent upon its content of any one, or a combination, of several compounds (vitamin B<sub>12</sub> (4) or the liver *Lactobacillus casei* factor (5), the yeast *L. casei* factor (5), the third *L. casei* factor (6), SLR factor (7), and thymine (1, 8)) and the treatment the material has undergone previous to assay. Bound forms of these compounds have been recognized repeatedly since the early work of Hutchings, Bohonos, and Peterson (9), who suggested that the norit eluate factor (another name for *L. casei* factor) was generally found associated with proteins.

After taka-diastase digestion was shown to be a good method for liberating bound "folic acid" by Cheldelin *et al.* (10), we used this procedure to help distinguish "folic acid" from vitamins B<sub>12</sub> and B<sub>11</sub>. We had previously used dilute ammonia under pressure to hydrolyze any ester of "folic acid" which might have been formed during alcohol separation procedures (11). Recently we showed that treatment with dilute acid or base increased the apparent "folic acid" content of certain materials (12); Binkley *et al.* (13) have used a specific enzyme to liberate vitamin B<sub>12</sub> from its conjugate in yeast and the Arkansas workers have used purified enzyme preparations to determine "preformed" and "potential" "folic acid" (14, 15). Stokes has shown that 0.05 N HCl and 0.05 N NaOH liberate a "thymine-like compound" from bacterial cells (16).

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<sup>1</sup> "Folic acid" is a term used to designate a substance necessary for the growth of *Streptococcus faecalis* R or *Lactobacillus casei* on certain defined media (1, 2). (*Streptococcus faecalis* R was previously called *Streptococcus lactis* R (3), American Type Culture Collection, No. 8043.)



Since the "folie acid" content of various materials is of particular interest to us in our attempt to evaluate this group of compounds in chick nutrition, we undertook further studies to determine the best method for liberating these active compounds.

### EXPERIMENTAL

The assay methods with *Streptococcus faecalis* R and *Lactobacillus casei* are those reported previously (1) with the exception that the incubation

TABLE I

"Folic Acid"-Deficient Media for *Streptococcus faecalis* and *Lactobacillus casei*

Constituent	Amount per 10 tubes	
	<i>S. faecalis</i>	<i>L. casei</i>
Glucose.....	1 gm.	2 gm.
Casein (acid-hydrolyzed).....	0.5 "	0.5 "
Dipotassium phosphate.....	0.5 "	0.2 "
Sodium acetate.....	0.2 "	0.6 "
Speakman's Salts B (17).....		0.5 ml.
<i>l</i> -Tryptophane*.....	30 mg.	10 mg.
<i>l</i> -Cystine.....	10 "	10 "
Asparagine.....		10 "
Adenine sulfate.....	1 "	1 "
Guanine hydrochloride.....	1 "	1 "
Xanthine.....	1 "	1 "
Uracil.....		1 "
Thiamine hydrochloride.....	20 $\gamma$	20 $\gamma$
Riboflavin.....	20 "	20 "
Nicotinic acid.....	60 "	60 "
Pyridoxine hydrochloride†.....	120 "	120 "
Calcium pantothenate.....	40 "	40 "
Biotin (free acid).....	0.04 "	0.04 "
<i>p</i> -Aminobenzoic acid.....		10 "
Water to.....	50 ml.	50 ml.

\* When *l*-tryptophane is unavailable, twice this amount of *dl*-tryptophane is used.

† Autoclaving this large amount of pyridoxine in the media gives adequate amounts of pseudopyridoxine. Since pyridoxal and pyridoxamine are becoming available (Merck and Company, Inc.), we now use 0.2  $\gamma$  of pyridoxamine per ten tubes.

period was generally extended to 20 hours as a matter of convenience. The composition of the media employed is given in Table I. Turbidimetric methods were used for clear samples and pH curves were run in the case of turbid samples. The final activity in all cases is reported as micrograms of vitamin B<sub>6</sub> (a standard having a potency of 80,000); this does not imply that all the activity in the sample was due to vitamin B<sub>6</sub>. Each value re-

ported is the average of the results of one to ten separate assays, each consisting of four tubes containing graded levels of the sample.

### Procedures

Cellular samples were homogenized in the Waring blender for 5 minutes before being treated.

*Taka-diastase Treatment (Adapted from Method of Cheldelin et al. (10))*—20 mg. of taka-diastase in 1 ml. of 1 per cent sodium acetate buffer were added to 1 gm. of sample in 8 ml. of buffer at pH 4.5 to 4.7 and incubated under toluene for 24 hours at 37°. The solution was then neutralized and assayed directly.

*Treatment with Laskowski Enzyme Obtained from Chicken Pancreas*—This procedure is based on the recommendations of M. Laskowski (private communication). 1 unit<sup>2</sup> of enzyme was added to 20 ml. of a solution containing either 100 mg. of a crude material or 20 mg. of a concentrate and incubated at pH 8 (no buffer) for 24 hours at 37° under toluene. The solution was assayed directly.

*Treatment with Other Enzymes*—0.15 mg. of the enzyme to be studied was added to 3 mg. of liver preparation, No. 214H (12) (equivalent to 5 gm. of starting material), in 25 ml. of 1 per cent buffer solution and incubated under toluene at 37° for 24 hours. The solution was then neutralized, autoclaved 15 minutes at 15 pounds pressure, and assayed.

*Acid Hydrolysis*—100 mg. of a concentrate or 1 gm. of a natural material was autoclaved in 20 ml. of 1 per cent acetate (sodium) buffer (pH 4.0) for 12 hours at 15 pounds pressure, neutralized, and assayed.

*Alkaline Hydrolysis*—100 mg. of a concentrate or 1 gm. of a natural material was autoclaved in 20 ml. of either 2 N base for 30 minutes or 0.1 N potassium hydroxide for 1 hour. The solution was then neutralized and assayed.

*Autolysis*—100 mg. of sample were placed in 20 ml. of water and incubated under toluene for 24 hours at 37°.

### Results

A preliminary report (12) on studies with liver Preparation 214H and yeast indicated that treatment with mild acid (pH 3 or 4) or base (2 N) gave the greatest increase in "folic acid" activity. Taka-diastase hydrolysis (pH 4.5) produced no greater increase in the apparent vitamin B<sub>12</sub> content than the same treatment without taka-diastase.

Preparation 214H was treated with many available enzymes and the

<sup>2</sup> 1 unit is that amount of enzyme which will produce the equivalent of 0.5  $\gamma$  of vitamin B<sub>12</sub> (potency 80,000) per hour for 4 hours when incubated in 11 ml. at 37° with 200 mg. of Difco yeast extract (15).

results (Table II) indicate that none of them gave a value higher than that obtained after acid hydrolysis.

Further study showed that heat (Fig. 1, *A*) aids greatly in the non-enzymatic liberation of compounds in the folic acid group. Temperatures higher than 122° were not tried, since they seemed impractical. The lower values obtained with concentrations of sodium hydroxide above 1 *N* (Fig. 1, *B*) may be due to specific destruction by sodium ions or possibly a toxicity to the bacteria. This effect is not noted with the concentrations of potassium hydroxide used.

TABLE II  
*Effect of Enzyme Treatment on Apparent Vitamin B<sub>12</sub> Content of Liver Preparation 214H\**

Sample No.	Treatment	pH	Buffer	Vitamin B <sub>12</sub> per gm.	
				<i>Streptococcus faecalis</i>	<i>Lactobacillus casei</i>
				$\gamma$	$\gamma$
1	None			0.06	0.30
2	Autoclaving, 12 hrs.	4.0	Acetate	0.63	0.80
3	Clarase	4.7	"	0.15	0.25
4	Papain	5.0	"	0.07	0.23
5	Pepsin	2.1	Chloride	0.13	0.45
6	Mylase P	4.9	Acetate	0.06	0.25
7	Pancreatin	8.2	Carbonate	0.06	0.17
8	Ficin	5.0	Acetate	0.08	0.18
9	5-Nucleotidase + hyaluronidase†	7.0	Phosphate	0.08	0.30

\* For the procedure see "Treatment with other enzymes."

† Obtained from horse semen through the cooperation of Dr. H. A. Lardy.

When the non-enzymatic liberation of activity was studied with respect to time (Fig. 1, *C*), it was found that autoclaving at pH 2 soon caused more destruction than liberation, while autoclaving at pH 3 for 1 to 6 hours gave acceptable results and pH 4 for 6 hours appeared to be the best method. Autoclaving the sample in 2 *N* base for half an hour gave high values; longer periods were not effective. Again it was noted that sodium hydroxide was less desirable than potassium hydroxide.

Further studies of the effect of time on the liberation of activity by autoclaving a vitamin B<sub>10</sub> and B<sub>11</sub> concentrate (Fig. 1, *D*) and Difco yeast extract (Fig. 1, *E*) indicated that autoclaving at pH 4 for 12 hours gave excellent results. Various other alkaline treatments gave values lower than those indicated. Consequently autoclaving at pH 4 for 12 hours was used routinely on more than 50 liver preparations. It is of interest to note that

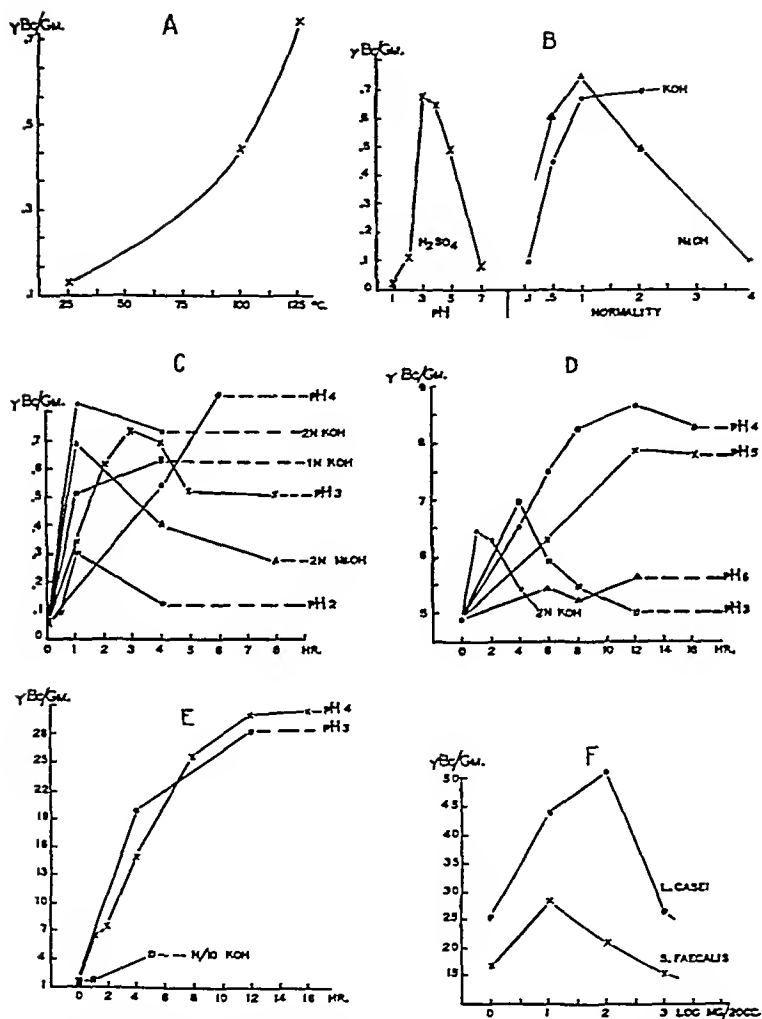


FIG. 1. Studies on the non-enzymatic liberation of vitamin B<sub>12</sub> activity from liver preparations and Difco yeast extract. *A*, effect of temperature on the response of *Streptococcus faecalis* to Preparation 214H treated at pH 3 for 4 hours; *B*, effect of [H<sup>+</sup>] on its response after autoclaving 4 hours at 122°; *C*, effect of time on its response after autoclaving at 122°; *D*, effect of time on the response of *S. faecalis* to Preparation 215 after autoclaving at 122°; *E*, effect of time on the response of *S. faecalis* to Difco yeast extract after autoclaving at 122°; *F*, effect of concentration on the response measured after autoclaving at 122° for 12 hours at pH 4.

TABLE III  
Effect of Different Hydrolysis Methods on Apparent Vitamin B<sub>6</sub> Content of Materials

The values are given in micrograms of vitamin B<sub>6</sub> per gm.

Sample	No treatment		pH 4, 12 hrs., 15 pounds pres- sure		Enzyme*		0.1 N KOH, 1 hr., 15 pounds		2 N KOH, 1/2 hr., 15 pounds		Taka-diastase, pH 4.6		Autoly- sis, 48 hrs., 37°	
	S. f.†	L. c.†	S. f.	L. c.	S. f.	L. c.	S. f.	L. c.	S. f.	L. c.	S. f.	L. c.	S. f.	L. c.
1. Ham (fresh) ..	0.006	0.03	0.03	0.05	0.09	0.09	<0.01	0.02	<0.01	0.01	0.15	0.18	0.07	
2. Beef round...	0.07	0.06	0.06	0.07	0.07	0.04	0.04	0.05	0.09	<0.005	0.15	0.14		
3. Chick kidney	0.47	0.43	0.47	0.44	0.49	0.45	<0.05	<0.05	0.34	0.40	2.6	1.2		
4. " liver.	1.4	1.7	1.6	2.1	3.0	2.6	<0.4	0.6	0.7	1.3	4.2	3.8	2.1	2.3
5. Pork liver powder..	24	15	19	19	19	15								
6. Yeast (Fleischmann).	0.47	0.70	6.8	7.6	1.4	1.4	<0.4	1.0	4.6	5.9	0.6	0.9	2.3	
7. " (Anheuser- Busch) . . .	0.25	0.33	3.5	8.7	6.2	6.6		6.0			2.3			
8. Yeast extract (Difco)	1.5	4.6	25	48	64	93	<10		20		<10		7	
9. Grass juice powder .	12	20	8.0	11.3	11.0	17	7.8	19	<2	12	11	20		
10. Milk (skim)... .	0.009	0.007	0.006		0.004	0.008			<0.004		0.005			
11. Beet . . . . .	0.05	0.05	0.08	0.03	0.08	0.07	<0.02	<0.02	<0.02	<0.02	0.28	0.23		
12. Wheat . . . . .	0.30	0.43	0.22	0.24	0.20	0.42	0.10	0.20	0.10	0.20	0.55	0.90		
13. Orange juice.....	<0.04	<0.04	0.10	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	<0.04	0.20	0.30		
14. Preparation 213B†	0.03	0.43	0.43	0.79	0.91	1.2								
15. " 215§.....	5.0	7.4	8.6	5.6	4.0	3.5								
16. " 255A  ...	0.007	0.07		0.40	0.08	0.54					0.05	0.20		
17. Lactobacillus casei factor .....	10,000	40,000												

\* Obtained from M. Laskowski (see the text).

† S. f. = *Streptococcus faecalis* R; L. c. = *Lactobacillus casei*.

‡ Preparation 213B was obtained by the procedure used for Preparation 233D (18).

§ Preparation 215 was made in the manner described for the Super Filtrate eluate (18).

|| Preparation 255A is the acid (pH 3)-alcohol (90 per cent) filtrate of Preparation 249E (18).

values with *Lactobacillus casei* were consistently higher than those with *Streptococcus faecalis*, although the values for acid-treated liver preparations showed a greater increase above their original value in the case of *S. faecalis* than in the case of *L. casei*. In fact more values decreased than increased for *L. casei*, while 60 per cent of the *S. faecalis* values increased more than 2 times.

The concentration of dry matter in the acid hydrolysis mixture was found to be of significance for values obtained with both *Streptococcus faecalis* and *Lactobacillus casei* (Fig. 1, *F*). The optimum concentration for Difco yeast extract appears to be around 10 to 100 mg. per 20 ml.

Through the courtesy of M. Laskowski we were able to test one of his enzyme preparations. When the results obtained with this enzyme were compared with results of other treatments, it was found (Table III) that the Laskowski enzyme gave higher values for some materials (see Samples

TABLE IV  
Variation in Assay of Difco Yeast Extract

Treatment	Vitamin B <sub>12</sub> content		
	<i>Streptococcus faecalis</i> R	<i>Lactobacillus casei</i>	No of assays
	$\gamma$ per gm	$\gamma$ per gm	
None.....	1-3	1-15	8
pH 3, 4 hrs., autoclaved	14-88	23-140	3
" 4, 12 " "	21-48	44-110	9
Laskowski enzyme, 24 hrs.	31-90	41-145	5

8 and 14), while acid treatment gave higher values for others (Samples 6, 15, and 16). Other materials gave no increase with either acid or enzyme (Samples 9 and 10). It is of considerable interest to note that the third *Lactobacillus casei* factor gave an increase in activity upon acid hydrolysis. This result substantiates the report of Day *et al.* (19). The data also indicate that treatment with 0.1 N potassium hydroxide gave poor results in most cases and 2 N potassium hydroxide treatment was not a good general procedure. Autolysis worked well for samples such as fresh liver, but poorly for yeast extract or ham. Combinations of various treatments, such as acid or base preceded or followed with enzyme treatment, gave lower results than a single treatment.

Although Difco yeast extract is an excellent source of potential vitamin B<sub>12</sub> activity, values obtained with it by various treatments (particularly the *Lactobacillus casei* values) are so variable that its vitamin B<sub>12</sub> content is difficult to determine (Table IV). This may be due to a delicate balance between liberation and destruction.

Incubation of a sample of Anheuser-Busch yeast with xanthopterin, according to the method of Wright and Welch (20), gave no higher values than incubation of the yeast without the xanthopterin. The increase observed may be attributed to the dilute acid (pH 4.5) used. Therefore, conclusions drawn from uncontrolled experiments showing the conversion of xanthopterin to "folic acid" (21, 22) are open to question. Wright *et al.* have recently shown (23) that xanthopterin or large amounts of neutral salts added to rat liver during incubation cause a definite increase in the "folic acid" content. This effect was attributed to a protective action against the destruction of "folic acid" after its liberation.

#### DISCUSSION

Although preliminary studies indicated that taka-diestase treatment was of little value for liberating compounds in the folic acid group from such materials as yeast, liver fraction L, or certain preparations made from them, later work indicates that taka-diestase treatment is generally superior to other methods of liberation for samples such as meat, grains, fruit, or vegetables. Evidently samples of yeast or liver require special treatment in order to determine the maximum apparent vitamin B<sub>12</sub> content. The fact that different treatments are required to give maximum values for different materials may indicate that the compounds in the folic acid group are bound in natural materials by different chemical unions. However, since dilute acid, dilute base, 2 N base, or taka-diestase all gave some liberation of "folic acid" activity, it seems probable that most of the liberation is due to hydrolysis.

Since the standard crystalline vitamin B<sub>12</sub> was found to undergo some destruction when autoclaved in dilute acid or base for 4 or 12 hours, the methods used here probably determine the optimum balance between liberation and destruction. Thus it is seen that hydrolysis at pH 3 or in 2 N base gave decreasing values after a certain time limit. It was also shown that liberation at pH 2 was impractical, probably because more destruction than liberation occurred, and pH 5 or pH 6 did not give conditions drastic enough for maximum liberation of the activity.

It was assumed that these methods involve only a liberation of bound forms of compounds in the folic acid group. That this is not entirely true may be seen from the increased activity obtained with crystalline third *Lactobacillus casei* factor; thus it is possible that one compound in the group may be changed into another more active compound.

#### SUMMARY

No one method can be prescribed to attain maximum "folic acid" values in all types of materials. Taka-diestase digestion gives maximum

values for many foodstuffs. The Laskowski enzyme, prepared from chicken pancreas (15), works well on certain materials such as Difco yeast extract. Autoclaving at pH 4 for 12 hours under 15 pounds pressure gives the highest "folic acid" values for certain liver preparations.

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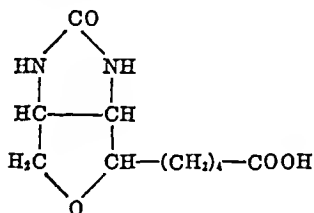
# THE MICROBIOLOGICAL ACTIVITY OF *dl*-OXYBIOTIN AND RELATED COMPOUNDS\*

By THEODORE WINNICK, KLAUS HOFMANN, FRANCIS J. PILGRIM,  
AND A. E. AXELROD

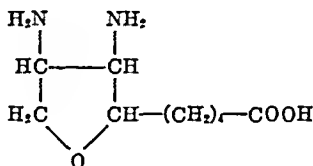
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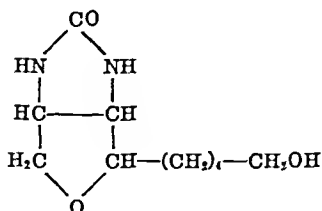
Preliminary studies on the biological activity of *dl*-oxybiotin (I) for microorganisms (1) and for higher animals (2) have been described recently. During the course of the oxybiotin synthesis (3, 4), a number of closely related compounds, such as *dl*-*cis*-3,4-diamino-2-tetrahydrofuranvaleric acid (II), *dl*-hexahydro-2-oxo-1-furo-(3,4)-imidazole-4-pentanol (III), and *dl*-hexahydro-2-oxo-4-methyl-1-furo-(3,4)-imidazole (IV), were prepared.



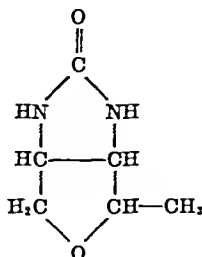
(I)



(II)



(III)



(IV)

The present communication describes in detail the microbiological activity of *dl*-oxybiotin and compounds (II), (III), and (IV) for a number of different organisms. In addition, a study of the ability of avidin to combine with these substances is presented.

\*The authors wish to express their appreciation to the Buhl Foundation and to Hoffmann-La Roche, Inc., for their generous support of this study.

### Methods and Materials

The ability of *dl*-oxybiotin and the compounds mentioned above to replace biotin as a growth factor for the following microorganisms was determined: *Lactobacillus arabinosus* (5), *Lactobacillus casei*, *Rhizobium trifolii* (6), and *Saccharomyces cerevisiae* (7). For the studies with *Lactobacillus casei*, the medium of Wright and Skeggs (5) was used, with the addition of 5  $\gamma$  of folic acid and 0.1 gm. of asparagine per liter of medium. The folic acid was furnished as a concentrate prepared from liver fraction L (Wilson) by the method of Hutchings *et al.* (8), and was determined by the procedure of Teply and Elvehjem (9), with a vitamin B<sub>6</sub> reference standard.

The avidin used in these studies was a crude concentrate. Solutions of the avidin were assayed with pure biotin and their activity expressed in avidin units (10). 1 avidin unit represents the amount of avidin which inhibits 1  $\gamma$  of biotin. In the inhibition experiments, the amount of avidin required completely to inhibit a quantity of growth factor, *employed at the level of half maximum growth*, was determined.

The activities of the various compounds tested were compared on a weight basis.

### Results

A comparison of the growth-promoting activities of *d*-biotin and *dl*-oxybiotin for *Lactobacillus arabinosus* and *Saccharomyces cerevisiae* is given in Table I. It is seen that the activity of *dl*-oxybiotin is one-half that of *d*-biotin for *L. arabinosus*. With this organism, the growth curves for the two compounds have identical shapes, and half maximum growth is attained with 0.4 millimicrogram of *d*-biotin and 0.8 millimicrogram of *dl*-oxybiotin. The activity of *dl*-oxybiotin for *L. casei* is approximately 80 per cent that for *L. arabinosus*; 1.0 millimicrogram gives half maximum and 5.0 millimicrograms give maximum growth.

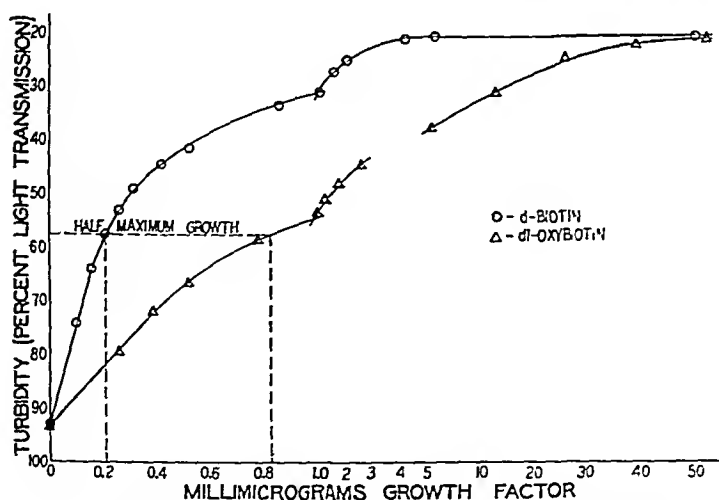
In the case of *Saccharomyces cerevisiae* (Fig. 1), the growth curves with *d*-biotin and *dl*-oxybiotin have somewhat different shapes. Half maximum growth is obtained with approximately 0.84 millimicrogram of *dl*-oxybiotin and 0.21 millimicrogram of *d*-biotin. However, in order to obtain maximum growth, 50 millimicrograms of *dl*-oxybiotin and only 5 millimicrograms of *d*-biotin are required. Thus, the relative activities differ considerably for different portions of the growth curves.

*dl*-Oxybiotin can replace biotin as a growth factor for *Rhizobium trifolii*. Half maximum growth is obtained with 0.09 millimicrogram of *d*-biotin and 0.75 millimicrogram of *dl*-oxybiotin. As in the case of *Saccharomyces cerevisiae* the growth curves for the two compounds have different shapes, and approximately 80 times more *dl*-oxybiotin than *d*-biotin are required

TABLE I

Comparison of Activities of Oxybiotin and Its Derivatives with That of Biotin

Test organism	Compound tested	Half maximum growth		Maximum growth	
		Amount	Relative activity	Amount	Relative activity
		millimicrograms		millimicrograms	
<i>Lactobacillus arabinosus</i>	<i>d</i> -Biotin	0.4	100	2.0	100
	<i>dl</i> -Oxybiotin	0.8	50	4.0	50
	<i>cis</i> -3,4-Diamino-2-tetrahydrofuranvaleric acid (II)	4000	0.01	20,000	0.01
	Hexahydro-2-oxo-1-furo-(3,4)-imidazole-4-pentanol (III)	300	0.13	1,500	0.13
<i>Saccharomyces cerevisiae</i>	<i>d</i> -Biotin	0.20	100	5	100
	<i>dl</i> -Oxybiotin	0.80	25	50	10
	" methyl ester	1.25	16	50	10
	<i>cis</i> -3,4-Diamino-2-tetrahydrofuranvaleric acid (II)	55	0.35	1,000	0.5
	Hexahydro-2-oxo-1-furo-(3,4)-imidazole-4-pentanol (III)	280	0.07	15,000	0.03

FIG. 1. Growth-promoting activities of *d*-biotin and *dl*-oxybiotin for *Saccharomyces cerevisiae*.

for maximum growth (*i.e.*, 1.0 millimicrogram of *d*-biotin and 80 millimicrograms of *dl*-oxybiotin).

The growth-stimulating activity of several oxybiotin derivatives has been determined, and the results are summarized in Table I.

The results of inhibition experiments with avidin are given in Table II.

TABLE II  
*Inhibition of Oxybiotin Derivatives by Avidin*

Compound	Avidin required to inactivate 1 $\gamma$ growth factor for <i>Saccharomyces</i> <i>cerevisiae</i>
	units
<i>d</i> -Biotin.....	1.0*
<i>dl</i> -Oxybiotin.....	1.0
“ methyl ester.....	1.2
Hexahydro-2-oxo-1-furo-(3,4)-imidazole-4-pentanol (III)....	1.4
<i>cis</i> -3,4-Diamino-2-tetrahydrofuranvaleric acid (II).....	†

\* By definition (reference activity).

† Not inhibited significantly by 100 units of avidin.

#### DISCUSSION

For *Lactobacillus arabinosus* the growth-promoting activity of *dl*-oxybiotin at half maximum growth is 50 per cent that of *d*-biotin; for *Lactobacillus casei*, 40; for *Saccharomyces cerevisiae*, 25; and for *Rhizobium trifolii*, 15 per cent.

It is a significant observation that the biological activity of biotin is only slightly affected by the substitution of oxygen for sulfur. There are two possible explanations for this behavior. Either the oxybiotin is active as such, or it is transformed into biotin by the organisms. Experiments reported in another paper (11) show that *Saccharomyces cerevisiae* and *Rhizobium trifolii* do not transform oxybiotin into biotin, and that oxybiotin possesses intrinsic biological activity. The sulfur atom therefore does not seem to be essential for the growth-promoting activity of the biotin molecule.

In the case of biotin, it has been established (12) that only one of the optical isomers, namely the *d* form, exhibits biological activity. It seems very probable that a similar situation exists in the case of oxybiotin. Accordingly, the activities given for the *dl* form of oxybiotin would represent the potency of only one of the enantiomorphs. For example, in the experiments with *Lactobacillus arabinosus*, the active form of oxybiotin would have biological activity equal to that of *d*-biotin. The resolution of *dl*-oxybiotin into its optical forms will provide the final confirmation of the above statements.

The importance of the carboxyl group for the biological activity is shown by the fact that hexahydro-2-oxo-1-furo-(3,4)-imidazole-4-pentanol (III), a derivative in which the carboxyl group of oxybiotin is replaced by a primary alcohol group, has only about 1/300 the activity of oxybiotin for both *Saccharomyces cerevisiae* and *Lactobacillus arabinosus*.

Whereas the methyl ester of biotin has the same yeast growth activity as biotin itself, the methyl ester of oxybiotin has only about 65 per cent the potency of the parent substance. In this connection it may be mentioned that hexahydro-2-oxo-4-methyl-1-furo-(3,4)-imidazole (IV), in which the valeric acid side chain of oxybiotin is replaced by a methyl group, has only about one-millionth the activity of *d*-biotin for *Saccharomyces cerevisiae*, *Lactobacillus arabinosus*, and *Lactobacillus casei*.

It has been shown that 3,4-diamino-2-tetrahydrothiophenevaleric acid, the substance resulting from the hydrolysis of the urea ring of biotin, possesses approximately 10 per cent of the activity of biotin for *Saccharomyces cerevisiae* (13). It is of interest that the analogous 3,4-diamino-2-tetrahydrofuranvaleric acid (II) derived from *dl*-oxybiotin is only about 1.5 per cent as active as *dl*-oxybiotin. For *Lactobacillus arabinosus* the activity of this compound is only 0.02 per cent that of *dl*-oxybiotin.

Oxybiotin and its methyl ester are inhibited by avidin. It is of interest that the pentanol derivative (III) is also inhibited by avidin. This observation suggests that the carboxyl group is not involved in the combination with avidin.

In agreement with the findings for biotin derivatives (13), the diamino-carboxylic acid (II) obtained by the hydrolysis of the urea ring of oxybiotin is not inhibited by avidin.

It has been established that the combination of biotin or its methyl ester with avidin is stoichiometric. A similar relationship has been found for oxybiotin and its methyl ester. Table II indicates that, with compounds of widely varying activities, approximately 1 unit of avidin is required to inactivate 1  $\gamma$  of growth substance. Thus, the pentanol derivative (III), with 1/1200 the activity of *d*-biotin, requires approximately 1700 times as much avidin for complete inhibition, at the level of half maximum growth.

The results in Table II make it appear likely that both the *d* and *l* forms of oxybiotin and its derivatives combine with avidin. If only one optical form were capable of combination, only half of the observed amounts of avidin would have been required.

We wish to thank Dr. J. A. Aeschlimann of Hoffmann-La Roche, Inc., for the avidin, Dr. D. Klein of The Wilson Laboratories for the liver fraction L, Parke, Davis and Company for the vitamin B<sub>7</sub> reference standard, and Merck and Company, Inc., for synthetic vitamins.

## SUMMARY

1. The activity of *dl*-oxybiotin compared to that of *d*-biotin at half maximum growth and expressed on a percentage basis is *Lactobacillus arabinosus* 50, *Lactobacillus casei* 40, *Saccharomyces cerevisiae* 25, and *Rhizobium trifolii* 15 per cent, respectively.

2. The hydrolysis of the urea ring in oxybiotin results in almost complete inactivation for *Saccharomyces cerevisiae* and *Lactobacillus arabinosus*. Replacement of the carboxyl group in oxybiotin by a primary alcohol group decreases the activity for these two organisms to 1/300 that of the parent substance.

3. Inhibition experiments with avidin demonstrate that oxybiotin, its methyl ester, and its pentanol derivative all combine with avidin in approximately the same molecular proportions as does biotin. The diamino derivative of oxybiotin is not inhibited by avidin.

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## LETTERS TO THE EDITORS

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### INCORPORATION OF ACETATE CARBON INTO RAT LIVER GLYCOGEN BY PATHWAYS OTHER THAN CARBON DIOXIDE FIXATION\*

Sirs:

This communication presents direct evidence that the intact mammal can incorporate acetate carbon into liver glycogen by pathways other than  $\text{CO}_2$  fixation.

It has been reported that  $\text{C}^{13}$  entering rat liver glycogen by  $\text{CO}_2$  fixation could be detected only in positions 3 and 4 of the glucose molecule,<sup>1</sup> and that the  $\text{C}^{13}$  of carboxyl-labeled acetate, likewise, could be detected only in these positions.<sup>2</sup> Subsequent experiments have shown that these are also the only positions in which excess isotope appears in rat liver glycogen after feeding carboxyl-labeled propionate and butyrate.<sup>3</sup> By the position of the labeled carbon, therefore, it is impossible to exclude  $\text{CO}_2$  fixation as the pathway of conversion of these fatty acids to glycogen.

Buchanan *et al.*<sup>4</sup> from similar experiments, using acetate, propionate, and butyrate labeled with  $\text{C}^{11}$  in the carboxyl groups, inferred that acetate carbon enters liver glycogen only via  $\text{CO}_2$  fixation, but that an additional mechanism of conversion is involved in the cases of propionate and butyrate.

Our own results with the carboxyl-labeled acids indicated, when allowance was made for  $\text{CO}_2$  fixation, that not only the propionate and butyrate enter glycogen in a form other than  $\text{CO}_2$ , but also the acetate. The findings of Rittenberg and Bloch<sup>5</sup> in feeding experiments with carboxyl-labeled

\* Aided by grants from the Medical Research Fund of the Graduate School of the University of Minnesota, and the Rockefeller Foundation, Research in Biology and Medicine.

<sup>1</sup> Wood, H. G., Lifson, N., and Lorber, V., *J. Biol. Chem.*, **159**, 475 (1945).

<sup>2</sup> Lifson, N., Lorber, V., and Wood, H. G., *Federation Proc.*, **4**, 47 (1945).

<sup>3</sup> Wood, H. G., Lifson, N., and Lorber, V., unpublished.

<sup>4</sup> Buchanan, J. M., Hastings, A. B., and Nesbitt, F. B., *J. Biol. Chem.*, **150**, 413 (1943).

<sup>5</sup> Rittenberg, D., and Bloch, K., *J. Biol. Chem.*, **157**, 749 (1945).



acetate, when similarly interpreted, are in harmony with our results with acetate.

To investigate this last point directly, young male rats were fed by stomach tube 2.5 mm of isotopic acetate and 400 mg. of glucose per 100 gm. of body weight. The acetate contained 4.30 atom per cent excess  $C^{13}$  in each carbon atom. 2.5 hours later the animals were sacrificed and the liver glycogen isolated, hydrolyzed, and degraded.<sup>1</sup> The bacterial degradation products showed the presence of 0.25 atom per cent excess  $C^{13}$  at positions 3 + 4, 0.20 per cent at 2 + 5, and 0.14 per cent at 1 + 6. By chemical degradation, 0.29 per cent was found at position 3, 0.24 per cent at 6, and 0.25 per cent at 1 + 2 + 4 + 5. This contrasts with the distribution found when acetate labeled only in the carboxyl carbon had been fed: 0.10 to 0.14 per cent at 3 + 4, 0.01 to 0.02 per cent at 2 + 5 and 1 + 6.

These findings furnish direct evidence that some pathway other than  $CO_2$  fixation must have been involved in the conversion of acetate to glycogen. That feeding large amounts of acetate may have provided routes over which  $CO_2$  carbon could enter positions other than 3 and 4 in the glucose molecule is excluded by the experiments with carboxyl-labeled acetate, in which excess isotope was detected only in these positions. The positions in the glycogen of labeled carbon from both carboxyl and doubly labeled acetate are consistent with predictions from current metabolic schemes.

The fact that a long chain fatty acid (myristic) can give rise to acetyl groups in the intact organism has been demonstrated.<sup>6</sup> It has further been found that acetate, by routes other than  $CO_2$  fixation, may enter amino acids which are considered to be in biological equilibrium with carbohydrate intermediates.<sup>5</sup> These findings, taken together with the present results, indicate that glycogen carbon can be derived from long chain fatty acid carbon without passing through the  $CO_2$  stage. At least in this sense, fats can apparently be converted to carbohydrate in the intact mammal.

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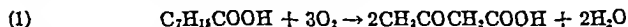
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<sup>6</sup> Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **155**, 243 (1944).

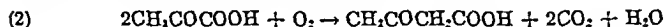
## FATTY ACID OXIDATION AND THE KREBS TRICARBOXYLIC ACID CYCLE

Sirs:

The author has described a washed rat liver enzyme suspension which oxidizes fatty acids in the presence of adenosine triphosphate.<sup>1</sup> On further study it has been found that these preparations oxidize octanoate to acetoacetate according to the equation



There is no endogenous oxidative activity. The preparation also catalyzes the reactions of the Krebs cycle. However, in the presence of malonate and the absence of 4-carbon dicarboxylic acids, the oxidation of pyruvate proceeds according to the equation



When fumarate is oxidized simultaneously with either octanoate or pyruvate in the presence of malonate, the yield of acetoacetate is greatly diminished and citrate accumulates. Acetoacetate itself is completely inert, forming no citrate in the presence of fumarate. The table shows a typical experiment.

It must be concluded that an oxidation product of fumarate (probably oxalacetate) is capable of condensing with reactive fragments derived not only from pyruvate oxidation but also from fatty acid oxidation to form citrate. Furthermore, since it has been shown that octanoate oxidation proceeds by loss of 2-carbon fragments, which may condense to form acetoacetate,<sup>2</sup> the data indicate that both fatty acid oxidation and pyruvate oxidation may result in 2-carbon fragments, which may react with each other to form acetoacetate or with oxalacetate to form citrate, depending on the supply of oxalacetate. The Krebs cycle therefore offers an explanation for "antiketogenesis."

The formation of citrate in these preparations differs from that observed by Hunter and Leloir<sup>3</sup> in kidney preparations, in that not acetoacetate but an acetoacetate precursor, probably a 2-carbon fragment, goes into the formation of citrate.

It would appear from these considerations that there exists a generalized condensation reaction between specific 2-carbon fragments and oxalacetate to form citrate, of which the Krebs condensation (pyruvate + oxalace-

<sup>1</sup> Lehninger, A. L., *J. Biol. Chem.*, in press.

<sup>2</sup> Weinhouse, S., Medes, G., and Floyd, N. F., *J. Biol. Chem.*, 155, 143 (1944).

<sup>3</sup> Hunter, F. E., and Leloir, L. F., *J. Biol. Chem.*, 159, 295 (1945).

tate  $\rightarrow$  "citrate") and the Breusch condensation<sup>4</sup> ( $\beta$ -keto acid + oxalacetate  $\rightarrow$  "citrate") are special cases, both acting through the formation of a 2-carbon fragment from the substrate (as has been suggested by several

Main compartment of Warburg vessel, 1.20 ml. of enzyme, 0.40 ml. of  $\text{MgSO}_4$  (0.005 M),\* 0.40 ml. of phosphate buffer, pH 7.7 (0.003 M), 0.40 ml. of sodium malonate (0.01 M), 0.40 ml. of adenosine triphosphate (0.0028 M), and 0.80 ml. of  $\text{H}_2\text{O}$ . Side arm, 0.20 ml. of  $\text{H}_2\text{O}$  or octanoate (0.001 M) or pyruvate (0.01 M) or acetoacetate (0.007 M) and 0.20 ml. of  $\text{H}_2\text{O}$  or fumarate (0.01 M). Center well, KOH. Gas phase, air; temperature, 25°. Equilibration period, 3 minutes, followed by addition of substrates and closing of taps. Time, 28 minutes. 22.4 c.mm. = 1 micromole.

Substrate	Fumarate	O <sub>2</sub> uptake	Change in acetoacetate	Citrate formation
		c mm	c mm	c mm
1. None	—	1	0	3.3
2. "	+	89	+3	29.8
3. Octanoate	—	216	+143	4.7
4. "	+	319	+57	58.4
5. Pyruvate	—	167	+158	4.5
6. "	+	259	+49	74.6
7. Acetoacetate	—	2	—4	3.9
8. "	+	84	—6	28.7

\* Figures in parentheses refer to the concentration of the component in the complete reaction medium.

authors). This interpretation attaches a broader significance to the Krebs cycle, the source of fragments for oxidation being (a) pyruvate oxidation, (b) fatty acid oxidation in the liver, and (c) acetoacetate oxidation in the extrahepatic tissues.

Butyrate, crotonate, vinyl acetate, *dl*- $\beta$ -hydroxybutyrate, aldol, and acetopyruvate are *not* intermediates in the formation of acetoacetate from octanoate nor are the following 2-carbon compounds (taken singly or in pairs): acetate, glycolate, glyoxylate, oxalate, acetaldehyde, ethanol, acetamide, glycine, acetyl phosphate, and acetyl pyrophosphate. Acetoin and diacetyl are *not* involved in the formation of acetoacetate. The search for the reactive 2-carbon fragments involved in acetoacetate and citrate formation is being continued.

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<sup>4</sup> Breusch, F. L., *Science*, 97, 490 (1943).

# THE DETECTION OF ACTIVATED CARBOXYL GROUPS WITH HYDROXYLAMINE AS INTERCEPTOR\*

Sirs:

Reliable tests for the formation of acyl phosphates in animal tissues had been frustrated until now by the presence of a powerful acyl phosphatase.<sup>1</sup> We have observed that the recently described fast reaction with hydroxylamine<sup>2</sup> may be used to trap acyl phosphate by immediate conversion into hydroxamic acid. The hydroxamic acid is eventually determined colorimetrically as described.<sup>2</sup> The maximum absorption of the iron compound was at 500 mμ, in agreement with the earlier described maximum of the iron compound of acethydroxamic acid.

When extracts of fresh or acetone-dried pigeon liver were incubated with mixtures of acetate and adenylyl pyrophosphate, considerable amounts of hydroxamic acid were formed, as shown in the experiment given in Table I. It indicates the transfer in liver tissue of phosphate from adenylyl pyrophosphate to the carboxyl group of acetate. A high affinity of the enzyme for acetate is indicated by occurrence of appreciable phosphorylation at 0.001 M acetate concentration. The reaction was best observed with pigeon liver preparations. Rat liver extracts did not so far give convincing results.

With the relatively low concentration of hydroxylamine used in the above experiment, acetate reacted rather specifically and only in conjunction with adenylyl pyrophosphate. Butyrate, octanoate, and decanoate were inactive, whether with or without adenylyl pyrophosphate. If, however, the concentration of hydroxylamine was raised, a general reactivity of fatty acids was observed which under these conditions became independent of the presence of an added phosphate donor. The experiment of Table II may serve as an example.

TABLE I

0.5 ml. of pigeon liver extract (1 gm. of acetone-dried liver extracted with 10 ml. of 0.02 M bicarbonate solution and centrifuged) was incubated for 60 minutes at 37° in test-tubes. The final volume was 1 ml. All samples contained 0.02 M neutralized hydroxylamine hydrochloride. The values are given in micromoles.

Acetate	10	10	3	1		10
Labile adenylyl pyrophosphorus	5	10	10	10	10	
Hydroxamic acid formed ..	1.39	1.76	1.77	1.35	0.90	0.35

\* This work was supported by a grant from the Commonwealth Fund

<sup>1</sup> Lipmann, F., *J. Biol. Chem.*, **160**, 173 (1945).

<sup>2</sup> Lipmann, F., and Tuttle, L. C., *J. Biol. Chem.*, **169**, 21 (1945).

TABLE II

0.5 ml. of rat liver homogenate (1 volume of liver homogenized with 2 volumes of Krebs' solution containing 0.03 M phosphate of pH 8) was incubated for 60 minutes at 37°. The concentration of hydroxylamine was here 0.4 M, the final volume 1.3 ml. 20 micromoles of the sodium salt of the indicated fatty acids were present per sample.

Fatty acid added.....	Acetic	Butyric	Octano-ic	Decano-ic	Dodecanoic.	None
Hydroxamic acid formed, micromoles.	0.72	1.79	2.50 2.82*	1.00	0.60	0.60 0.83*

\* With 10 micromoles of labile adenylyl pyrophosphorus.

This reaction was found, in either air or nitrogen, with extracts from rat and pigeon liver but not with that from rabbit brain, nor did controls without the enzymes show formation of hydroxamic acid. Of the tested fatty acids octanoate was the most reactive under these conditions, while acetate showed very little reactivity.

A study is in progress to investigate more thoroughly the mechanism of this quite abundant conversion of fatty into hydroxamic acids without addition of a phosphate donor. The small amounts of adenylyl pyrophosphate present in these extracts are certainly insufficient to explain the reaction.

The experiments reported here were largely motivated by the work of Lehninger<sup>3</sup> on a phosphorylation of fatty acids prior to oxidation in the liver.

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<sup>3</sup> Lehninger, A. L., *J. Biol. Chem.*, **157**, 363 (1945).

## THE FATE OF N<sup>1</sup>-METHYLNICOTINAMIDE IN MAN\*

*Sirs:*

Since the establishment of the fact that N<sup>1</sup>-methylnicotinamide (F<sub>2</sub>) represents the major known niacin derivative found in the urine of man normally and after doses,<sup>1</sup> numerous attempts have been made to base a quantitative estimate of the state of niacin nutrition on the determination of F<sub>2</sub> in the urine.<sup>2</sup>

Our recent observations, confirming those of Ellinger and Coulson,<sup>3</sup> have shown that in man (and in the rat) F<sub>2</sub> is not the final metabolite but is itself further metabolized into unidentified products. The table, representing a typical example from a large body of data, demonstrates that the excretion of extra F<sub>2</sub> is approximately the same after the ingestion of equivalent amounts of nicotinamide or of N<sup>1</sup>-methylnicotinamide, corresponding to 10 to 20 per cent of the ingested dose.

After single oral or intravenous doses of N<sup>1</sup>-methylnicotinamide the excretion of the three derivatives returned to control levels in the second 24 hours; after nicotinamide and after trigonelline the excretion of the extra methylated derivatives was completed in 2 days, about 80 per cent of the recovery value on the 1st, and 20 per cent on the 2nd day. That delayed excretion is not a significant factor is indicated by our observation in which three normal adults ingesting daily 500 mg. of nicotinamide for 25, 35, and 48 days excreted at the end of these periods 32, 27, and 27 per cent, respectively, of the ingested doses, practically all in the methylated form. The absence of any increase in the N. A. fraction after the ingestion of F<sub>2</sub> indicates that the unknown metabolic products are not of the nature of niacin derivatives which might have been obtained by the demethylation of the F<sub>2</sub>. That the methylnicotinamide is not appreciably transformed into trigonelline in the body by the removal of the amide group is also clearly shown in our data. The previously established fact of the stability in the body of ingested trigonelline is again borne out by the data in the table which are included for comparison. Intravenous administration of 1.63 milliequivalents of F<sub>2</sub> to subject B led to the recovery of 67 per cent in the

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<sup>1</sup> Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **150**, 395, 483 (1943). Sarett, H. P., *J. Biol. Chem.*, **150**, 159 (1943).

<sup>2</sup> Reviewed in *Nutr. Rev.*, **3**, 233 (1945).

<sup>3</sup> Ellinger, P., and Coulson, R. A., *Biochem. J.*, **38**, 265 (1944).

urine, indicating that 33 per cent was "destroyed" in the body outside of the intestinal tract.

In view of these facts, it is obvious that the excretion of  $F_2$  represents primarily the resultant of the rates of two or more metabolic reactions involving niacin: its methylation to  $F_2$  and the subsequent conversion of  $F_2$  to products as yet unknown. This may explain the inconsistencies and the difficulties found in our attempts and those of others to correlate the urinary excretion of  $F_2$  with the state of niacin nutrition.

*24 Hour Excretion of Niacin Derivatives by Two Normal Male Adults*

N. A., total niacin derivatives obtained by hydrolysis with 7 N HCl; Trig., the fraction including trigonelline and  $F_2$  as determined by the method of Perlzweig, Levy, and Sarett (*J. Biol. Chem.*, **136**, 729 (1940));  $F_2$ , as determined by the fluorometric method of Huff and Perlzweig (*J. Biol. Chem.*, **150**, 483 (1943)).

Oral dose	Subject A			Subject B		
	N. A.	Trig.	$F_2$	N. A.	Trig.	$F_2$
	mg.	mg.	mg.	mg.	mg.	mg.
Control . . . . .	1.26	12.8	11.7	1.3	12.5	11.0
200 mg. (1.63 m.eq.) nicotinamide	1.8	55.0	49.4	1.5	47	44
282 " (1.63 " ) N <sup>1</sup> -methylnicotinamide Cl . . .	1.2	61.0	51.6	1.2	44	37.2
282 mg. (1.63 m.eq.) trigonelline HCl	1.3	185.0	11.0	1.4	190	11.4

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# HYDROLYSIS OF PHENYLACETYLCHOLINE BY ESTERASES\*

Sirs:

In view of the essential rôle of acetylcholine (ACh) in the conduction of the nerve impulse, cholinesterase, the enzyme which has the function of removing the active ester by hydrolysis, is of great physiological interest.<sup>1</sup> A great number of esterases occur in the organism and it is not easy to distinguish between the esterase specific for ACh and other unspecified esterases, since the specificity in this case is only relative.

However, by testing a number of choline and non-choline esters, a pattern has been established which makes it possible to determine whether or not an enzyme is cholinesterase.<sup>2</sup> Also, it has been shown that a number of drugs differ significantly in their affinities for cholinesterase and for other esterases.<sup>3</sup> Even so, the distinction sometimes becomes difficult with the compounds available, especially in certain nerve tissues in which a mixture is present.<sup>4</sup> It appeared therefore desirable to look for other compounds which would be useful in clarifying the above situation.

A new compound of this kind has now been found in phenylacetylcho-

*Ratio of Hydrolysis of Phenylacetylcholine Chloride to Acetylcholine Chloride  $\times 100$   
(= Q) by Unspecified Esterases*

Q is always equal to 0 for cholinesterase.

Cholinesterase			Unspecified esterases			
Species	Tissue	ACh hydrolysis, CO <sub>2</sub> in 20 min. c.m.m.	Species	Tissue	ACh hydrolysis, CO <sub>2</sub> in 20 min. c.m.m.	Q
<i>Electrophorus electricus</i>	Electric organ	134	Guinea pig	Pancreas	241	11
		136			164	15
Ox	Nucleus caudatus	126	" "	Kidney	80	106
		135			74	104
Squid	Head ganglion	179	" "	Liver	38	810
					128	48
			Horse	Serum	138	40

\* This work was aided by a grant from the Josiah Macy, Jr., Foundation.

<sup>1</sup> Nachmansohn, D., in Harris, R. S., and Thimann, K. V., *Vitamins and hormones*, New York, 3, 337 (1945).

<sup>2</sup> Nachmansohn, D., and Rothenberg, M. A., *J. Biol. Chem.*, 158, 653 (1945).

<sup>3</sup> Nachmansohn, D., and Schneemann, H., *J. Biol. Chem.*, 159, 239 (1945).

<sup>4</sup> Nachmansohn, D., and Rothenberg, M. A., in preparation.



line.<sup>5</sup> The introduction of a phenyl group into the acetate makes the ester completely resistant to cholinesterase, whereas other esterases are able to split this compound (see the table). Great differences, however, are found as to the rates; the esterase of the pancreas splits the aromatic compound at a rate only 15 per cent of that at which ACh is split, whereas liver esterase splits it 8 times more rapidly than ACh. The variations in the rate of hydrolysis by the different esterases appear to be more marked than are found with other esters. Thus, the compound is valuable not only for a test of specificity but also emphasizes the existence of a great variety of esterases in the body. In spite of the similarity of the ester linkage, the esterases react so differently to a change in the chemical structure of the ester that it appears more justifiable to speak of unspecified esterases<sup>1</sup> rather than unspecific, thereby implying that the specific substrate is as yet unknown.

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# ISOLATION OF THE ACID-SOLUBLE NUCLEOTIDES OF LIVER

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In the course of studies on the acid-soluble phosphorus compounds of liver and kidney, it became evident that the fraction comprising the nucleotides represented a complex mixture (1). In an attempt to isolate the individual nucleotides, methods designed to minimize the breakdown of labile phosphorus compounds were employed. The isolation of adenylic acid and of a material representing a mixture of adenosine di- and triphosphate from rabbit and dog liver is reported in the present communication.

The following steps were taken in the isolation of the products. The organs were removed quickly from narcotized animals and put into a dry ice-ether mixture. In the trichloroacetic acid extract the nucleotides together with glutathione and an unknown nitrogen-free phosphorus compound were precipitated with mercuric acetate. After removal of the mercury, the barium salts were prepared and divided into a soluble fraction, containing adenylic acid and glutathione, and an insoluble fraction, containing adenosine triphosphate and the bulk of the adenosine diphosphate. From the soluble fraction free adenylic acid was isolated after a preliminary precipitation with phosphotungstic acid to separate it from glutathione. Adenosine triphosphate and the bulk of the adenosine diphosphate, present in the insoluble fraction of the barium salts, were isolated after repeated fractionation as silver salts from 0.2 N nitric acid to remove the nitrogen-free contaminant.

The preparations were analyzed for nitrogen by a Kjeldahl procedure, for adenine (2), and for easily hydrolyzable and total P (3). Determination of the total nucleotide phosphorus was performed with rattlesnake venom as a specific nucleotidase according to Hitchings (4). The content of adenosine triphosphate was assayed by three methods, (a) rattlesnake venom (4), (b) myosin (5, 6), and (c) washed lobster muscle (7).

## *Isolation Procedure*

For experiments on a preparative scale the livers of two or three rabbits or of one dog were removed under anesthesia, cut quickly into small chunks with shears, and dropped into a dry ice-ether mixture. 100 gm. portions of liver were ground in a Waring blender with 5 volumes of 5 per cent trichloroacetic acid for periods of 4 minutes. The resulting mixtures were

centrifuged and the supernatant fluids were filtered. The filtrate, more or less opalescent according to the amount of glycogen, was neutralized with 5 N NaOH, and 0.05 volume of a solution of 20 gm. of mercuric acetate in 100 cc. of 2 per cent acetic acid was added. After standing for several hours the precipitate was centrifuged, washed once with a 40-fold dilution of the mercuric acetate reagent, suspended in water, and decomposed with  $\text{H}_2\text{S}$ . The supernatant fluid of the mercury sulfide precipitate and the washings were freed of  $\text{H}_2\text{S}$  by aeration. They were opalescent and sometimes brownish in color, owing to the presence of glycogen and of finely dispersed mercuric sulfide. 1 volume of a 50 per cent solution of barium acetate and 0.2 volume of alcohol were added and the fluid was made alkaline to phenolphthalein by the addition of NaOH. 15 minutes later the precipitate was separated by centrifugation, redissolved in trichloroacetic acid, and reprecipitated as barium salt in the same manner as before. The isolation of adenosine diphosphate and triphosphate from the precipitate is described in Section 1, and of adenylic acid from the supernatant fluid in Section 2.

*Section 1. Isolation and Analysis of Mixture of Adenosine Di- and Triphosphate*—The precipitate of water-insoluble barium salts was dissolved in a small amount of 0.5 N  $\text{HNO}_3$ , and enough  $\text{H}_2\text{SO}_4$  was added to precipitate all the barium which was removed by centrifugation. The glycogen, which accompanied the nucleotides up to this point, was then precipitated by the addition of 0.66 volume of alcohol, and removed by centrifugation. The glycogen was dissolved and reprecipitated once more from a small volume of fluid. A few cc. of 5 N  $\text{AgNO}_3$  were added to the combined supernatant fluid which contained 40 per cent of alcohol by volume and which was 0.2 N with respect to nitric acid. The resulting precipitate was centrifuged, decomposed with  $\text{H}_2\text{S}$ , aerated, and the nucleotides were reprecipitated twice more from a 0.2 N nitric acid solution by the addition of 0.05 volume of  $\text{AgNO}_3$ . This fractionation as silver salt effected the removal of a nitrogen-free phosphorus compound occurring in liver. For analytical purposes the anhydrous alkaline barium salts were prepared. The yield of adenosine polyphosphate was about 100 mg. of barium salt per 100 gm. of liver.

#### *Analyses—*

Rabbit liver nucleotide, found,	N	9.4,	P	10.3,	Ba	33.2,	N:P	=	5:2.48
Dog liver nucleotide, " "	"	9.6,	"	9.7,	"	32.9,	"	=	5:2.29
$\text{C}_{10}\text{H}_{12}\text{O}_{13}\text{N}_5\text{P}_3\text{Ba}_2$ , calculated,	"	9.0,	"	12.0,	"	35.3,	"	=	5:3
$\text{C}_{10}\text{H}_{13}\text{O}_{10}\text{N}_5\text{P}_2\text{Ba}_{1.5}$ , " "	"	11.1,	"	9.8,	"	32.7,	"	=	5:2

The proportion of hydrolyzable phosphate was 60 and 54 per cent of the total for the rabbit liver and the dog liver preparations, respectively. The

purine nitrogen content amounted to 98 per cent of the total nitrogen. The purine of the nucleotides was identified as adenine by preparation of the picrate in 90 to 92 per cent yield, according to the procedure of Kerr (8). These adenine picrate crystals melted sharply at 289°. The picrate from the rabbit liver preparation was analyzed for nitrogen with the following results.

$C_{11}H_8N_4O_7$  (364.24). Calculated, N 30.8; found, N 30.7

Adenosine diphosphate was prepared from the nucleotide mixture of the rabbit liver by the use of washed lobster muscle (7) in 82 per cent yield. It was isolated by following closely the procedure described by Engelhardt (5). The anhydrous barium salt was analyzed with the following results.

$C_{10}H_{12}O_{10}N_5P_2Ba_{1.5}$	Calculated.	N 11.1, P 9.8, Ba 32.7, N:P 5:2
	Found.	" 10.4, " 9.4, " 33.2, " 5:2.04

50 per cent of the phosphate was hydrolyzable by heating for 15 minutes in  $\alpha$  acid at 100°. Analysis of this material with the enzyme of the rattlesnake venom and with myosin indicated the presence of 92 to 94 per cent of adenosine diphosphate, the rest being adenosine triphosphate.

### *Enzyme Experiments*

*Assay of Adenosine Triphosphate Content by Use of Rat Myosin*—This method is based on the fact that purified myosin acts as an adenosinetriphosphatase, converting adenosine triphosphate to adenosine diphosphate with the liberation of 1 mole of inorganic P per 1 mole of the triphosphate (Engelhardt). The myosin used in these experiments was prepared from rat muscle by the method of Bailey (6) with slight modifications. For the assay of adenosine triphosphate the reaction mixtures were prepared as follows: 1 cc. of myosin solution in 0.1 M glycine buffer of pH 9.1 and containing 0.1 to 0.3 mg. of protein nitrogen, and 0.1 cc. of 0.1 M  $CaCl_2$  solution were added to 2.5 cc. of a neutral substrate solution. The mixtures were incubated at 37° for 30 minutes, and the reaction was then stopped by the addition of 4 cc. of 20 per cent trichloroacetic acid. Water was then added to the volume of 10 cc. Control samples were set up by adding the trichloroacetic acid before the addition of the enzyme. The difference between the values for inorganic P before and after incubation represents one-third of the adenosine triphosphate content of the substrate. The enzyme was checked repeatedly with pure adenosine triphosphate from rabbit muscle, and with an adenosine diphosphate preparation, with results conforming to expectations.

*Assay of Adenosine Triphosphate Content by Use of Washed Lobster Muscle*—Lobster muscle washed repeatedly with 0.06 M KCl solution,

according to Lohmann (7), acts like myosin in splitting adenosine triphosphate. The experiments were performed in the manner described by him (7) and by Kiessling and Meyerhof (9). From test preparations of adenosine triphosphate about 10 per cent more inorganic P was liberated than was expected.

*Determination of Total Nucleotide P and of Adenosine Triphosphate Content by Use of Rattlesnake Venom*—The venom of the rattlesnake, *Crotalus adamanteus*, according to Hitellings (4) acts as a specific nucleotidase. Adenylic acid and adenosine diphosphate are hydrolyzed by the venom with complete liberation of their P as orthophosphate, while adenosine triphosphate is decomposed with the formation of 1 mole of pyrophosphate and 1 mole of orthophosphate. The inorganic pyrophosphate, representing two-thirds of the total P of adenosine triphosphate, may be hydrolyzed by heating in N acid for 15 minutes at 100°. The estimate of the inorganic pyrophosphate provides another measure of the adenosine triphosphate content of nucleotide preparations. By combined enzyme action and acid hydrolysis complete liberation of the P from nucleotides may be achieved. With test preparations the adenosine triphosphate content was found to be somewhat low by this method, since apparently some hydrolysis of the inorganic pyrophosphate takes place during the course of incubation. The estimates of the total nucleotide content, however, were found to be reliable. The experiments were performed as follows: 2 to 4 cc. of substrate solution containing about 0.3 mg. of nucleotide P were neutralized with a few drops of a 10 per cent  $\text{NaHCO}_3$  solution. 1 cc. of Michaelis buffer and 2.5 mg. of venom dissolved in 1 cc. of 0.9 per cent NaCl solution were added. The mixtures were incubated for 75 minutes at 38°, and the experiments were terminated by the addition of 4 cc. of trichloroacetic acid and of water to the volume of 10 cc. Inorganic P and easily hydrolyzable P were determined. It was assumed that 1.5 times the value for the easily hydrolyzable P corresponded to the adenosine triphosphate content, while the value for inorganic P at the end of 15 minutes hydrolysis in N acid represented the total amount of nucleotide phosphorus. Assay of adenosine triphosphate content of liver nucleotide polyphosphate preparation gave the following results, in per cent of total.

Liver	N:P ratio	Myosin	Venom	Lo bster muscle
Rabbit.....	48	54	51	60
Dog.....	29	39	36	48

The total nucleotide P value in each case corresponded closely to the value for total phosphorus.

*Section 2. Isolation of Adenylic Acid*—The supernatant fluid of the barium precipitate contained adenylic acid with about 15 per cent admixture of adenosine diphosphate in terms of purine nitrogen, but was free of adenosine triphosphate as judged by tests with rattlesnake venom and myosin. 3 volumes of 95 per cent alcohol were added to this supernatant fluid. The resulting precipitate, containing glutathione in addition to the nucleotides, was extracted three times with dilute acetic acid, a small residue being discarded. The barium in the combined extracts was removed with sulfuric acid, the solution was made 0.5 N with respect to sulfuric acid, and 0.2 volume of 20 per cent phosphotungstic acid in 0.5 N  $\text{H}_2\text{SO}_4$  was added. The mixture was kept ice-cold overnight. The precipitate was separated by centrifugation, washed twice with 2 per cent phosphotungstic acid, and was then dissolved in 50 per cent acetone. Baryta was added to the definitely alkaline reaction of phenolphthalein. The bulky precipitate of barium phosphotungstate was extracted several times with dilute baryta solution. An excess of 40 per cent lead acetate solution and 1 volume of alcohol was added to the combined extract. The lead precipitate was washed two times with 50 per cent alcohol, suspended in water, decomposed with  $\text{H}_2\text{S}$ , and a few drops of dilute  $\text{H}_2\text{SO}_4$  were added to remove small amounts of barium. The supernatant fluid of the lead sulfide combined with the washings was concentrated almost to a dryness *in vacuo* at a temperature below  $40^\circ$ . The contents of the flask were transferred with several small washings of hot water into a small tube. 3 volumes of acetone were added and the tube was left in a refrigerator overnight. The adenylic acid was recrystallized three times from 50 per cent acetone. Small amounts of phosphotungstic acid which escaped the precipitation with barium were removed in the process of recrystallization from acetone. The nucleotide was recrystallized twice more from hot water, and then washed with alcohol and ether. The resulting white product melted at  $196^\circ$ , uncorrected. The yield of adenylic acid was about 10 mg. per 100 gm. of liver. Analysis of the anhydrous material gave the following results.

$\text{C}_{10}\text{H}_{14}\text{O}_7\text{N}_5\text{P}$  (347.3). Calculated, N 20.2, P 8.9; found, N 20.3, P 8.8

#### DISCUSSION

The presence in the liver of acid-soluble nucleotides less highly phosphorylated than adenosine triphosphate appeared likely when it was found that the easily hydrolyzable P represented only 45 per cent of the P which is precipitated by mercuric acetate (1). This supposition was supported by the finding that the concentrations of purine nitrogen and of pentose in the trichloroacetic acid extracts were in excess of those calculated for

adenosine triphosphate from the data on the easily hydrolyzable P. Differences between fed and fasted rats indicated that the presumed mixture of nucleotides was subject to variations under different conditions of the metabolism of the liver. The isolation of adenylic acid and of a mixture of adenosine di- and triphosphate, here reported, supports the conclusion reached from the earlier experiments. However, in view of the chemical lability of adenosine triphosphate and of the presence of powerful enzyme systems in liver, the possibility should be examined that the less highly phosphorylated nucleotides were products of chemical or enzymatic breakdown. As far as adenosine diphosphate is concerned this possibility appears remote, considering the large amounts found and the fact that this substance is not known to be a product of the chemical breakdown of adenosine triphosphate. The chance of such an origin of adenylic acid may be denied with less assurance, since this compound can be formed both enzymatically and by chemical breakdown of adenosine triphosphate at alkaline reaction. The extent of the enzymatic breakdown was kept at a minimum by prompt freezing of the organs after removal. The success of this measure was indicated by the close correspondence of the figures for inorganic and easily hydrolyzable P between extracts of small and of large tissue samples. In the fractionation of the barium salts, under conditions which favor the breakdown of adenosine triphosphate to adenylic acid, the exposure to alkaline reaction was as brief as possible with careful control of the temperature. No significant formation of adenylic acid took place during the isolation procedure, as judged from the analyses by the method of Hitchings, which were carried out at every step.

The acid-soluble nucleotides in various tissues appear to vary both in concentration and in degree of phosphorylation. In muscle, brain (8), and in erythrocytes (10, 11) almost the entire nucleotide content is accounted for as adenosine triphosphate. In liver, in heart muscle (12), and in kidney (unpublished work), on the other hand, less highly phosphorylated nucleotides can be demonstrated. The factors responsible for such differences are at the present time a subject for speculation only.

#### SUMMARY

The acid-soluble nucleotides of liver comprise a mixture of compounds representing different degrees of phosphorylation of adenylic acid. The isolation of adenylic acid and of a mixture of adenosine triphosphate and adenosine diphosphate from trichloroacetic acid extracts of liver is reported.

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# THE DISTRIBUTION OF ACID-SOLUBLE NUCLEOTIDES IN THE LIVERS OF RATS, FED AND FASTING

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Previous studies have indicated that the acid-soluble adenine nucleotides of liver represent a mixture of compounds of varying degree of phosphorylation (1). Large differences in the concentration of easily hydrolyzable P between fed and fasting rats in the face of constant concentrations of purine N and stable mercury-precipitable P indicated that the degree of phosphorylation of the mixture of nucleotides varied with the nutritional status of the animals (2).

The present communication deals with an attempt to define more clearly the composition of the nucleotide mixture and its changes during fasting by a system of analyses that permitted an estimate of the individual nucleotides. For this purpose the trichloroacetic acid extract of liver was precipitated with mercuric acetate. On this precipitate, which contains all the nucleotides and some non-nucleotide P compounds as well, analyses were performed for easily hydrolyzable and total P, for nucleotide P by means of the specific nucleotidase of rattlesnake venom (3), and for adenosine triphosphate by the use of the adenosinetriphosphatase of myosin (1). The values for adenosine diphosphate and adenylic acid were calculated from these data. Determinations of the purine nitrogen and of pentose, which furnished independent estimates of the nucleotide content, were performed on the mercury precipitate. The inorganic and easily hydrolyzable P were determined in the trichloroacetic extract for purposes of comparison with earlier experiments.

## Material and Methods

Male rats weighing  $220 \pm 45$  gm. from the stock of Sprague-Dawley, Inc., were used. Rats designated as fed had free access to food at all times, while the food was withdrawn for either a 24 or a 48 hour period from the fasted animals. The analytical results for all fasted animals were combined, since they were not significantly different. The preparation of the trichloroacetic acid extracts from liver has been described elsewhere (4). The determination of the inorganic, the easily hydrolyzable, and the total P in the trichloroacetic acid extract has also been described previously (2).

*Preparation and Analysis of Mercury Precipitate*—A 50 cc. sample of trichloroacetic acid extract was transferred to a 65 cc. centrifuge tube, neutralized with 5 N and N NaOH, and 3 cc. of mercuric acetate reagent (20 per cent mercuric acetate in 2 per cent acetic acid) were added. After standing for 1 hour in the refrigerator the material was centrifuged, the supernatant fluid was discarded, and the precipitate was washed once by suspension in 5 cc. of water and recentrifugation. The precipitate was suspended once more in 4 cc. of water, and the mercury was precipitated with a current of  $H_2S$  gas. The mercuric sulfide precipitate was removed by centrifugation, washed once with a small volume of water, and the combined supernatant fluids, after aeration to eliminate the  $H_2S$ , were made to a definite volume. All the steps were carried out in a cold room or in an ice bath.

Determinations of the distribution of P, the content of purine N, and of pentose were performed on the solution of the mercury precipitate as follows.

*Distribution of P in Mercury Precipitate*—The subsequent fractions were determined: inorganic P, easily hydrolyzable P, total P, P liberated by action of myosin, and that by combined action of rattlesnake venom and 15 minutes hydrolysis in N acid at  $100^\circ$ . Based on these data the following P fractions were defined.

- (1) Total organic P = total P minus inorganic P
- (2) " nucleotide P = inorganic P after incubation with rattlesnake venom and acid hydrolysis (minus inorganic P before incubation and hydrolysis)
- (3) Non-nucleotide P = total organic P minus total nucleotide P
- (4) Stable nucleotide P = total nucleotide P minus easily hydrolyzable P
- (5) Adenosine triphosphate = (inorganic P after action of myosin)  $\times 3$
- (6) " diphosphate =  $2 \times$  (easily hydrolyzable P -  $2 \times$  inorganic P after myosin)
- (7) Adenylic acid = total nucleotide P minus adenosine tri- and diphosphate

The preceding equations are based on these considerations: Rattlesnake venom according to Hitchings (3) attacks specifically nucleotides, splitting adenylic acid and adenosine diphosphate completely, and adenosine triphosphate with the liberation of 1 mole of orthophosphate and 1 mole of pyrophosphate. After incubation of the material with venom and subsequent hydrolysis for 15 minutes in N acid at  $100^\circ$ , the entire phosphorus of the nucleotides is obtained in inorganic form. The difference between the total organic P and the nucleotide value, constituting the non-nucleotide P in the mercury precipitate, is in part accounted for by a nitrogen-free unidentified phosphorus compound. The *easily hydrolyzable* P represents two-thirds of the adenosine triphosphate and one-half of diphosphate, while only a negligible amount of adenylic acid is decomposed

under these conditions. Myosin as a specific adenosinetriphosphatase splits one of the two easily hydrolyzable phosphate groups of the adenosine triphosphate, converting it to adenosine diphosphate. From these data the equations listed above can easily be derived. The fraction most inaccurately determined is adenylic acid, whose value is calculated as a difference from three different determinations; namely, venom action, myosin incubation, and acid hydrolysis. All values were corrected for the amounts of inorganic P in the precipitate, which averaged 4 mg. per 100 gm. of liver in fed, and 8 mg. per 100 gm. in fasted rats.

The determination of the purine nitrogen in the nucleotides was carried out according to the procedure of Kerr and Blish (5) and Kerr (6) modified as follows. After acid hydrolysis and precipitation of the nucleotides with copper hydroxide, 1 cc. of 5 per cent sodium citrate was added to the precipitate and the mixtures were heated for 15 minutes in a boiling water bath. Then 1 cc. of  $N H_2SO_4$  was added and the precipitation with sodium bisulfite was carried out as in the original method. This modification was necessitated by the presence of large amounts of glutathione in liver. The average yield of purine  $N$  by this procedure was 94 per cent, and all data were corrected accordingly.

The determination of pentose in the mercury precipitate was carried out by the method of Mejbaum (7), modified by the use of 2 cc. of 0.05 per cent solution of  $FeCl_3$  in concentrated  $HCl$  instead of 1 cc. of 0.1 per cent solution, and by extending the heating time to 30 minutes. The samples were diluted to 7 cc. with water and read in the Evelyn colorimeter with a 6 cc. aperture and Filter 660. The purity of the color was always checked by readings with Filter 520. A reference curve was prepared with myo-adenylic acid as a standard, which yields about 20 per cent more color than arabinose. Even small amounts of glycogen interfere seriously with this method. Therefore, glycogen was removed by the addition of an equal volume of alcohol before the determination was carried out.

### Results

In Table I are summarized the results of the analyses on the mercury precipitate and the trichloroacetic acid extract of the livers of fed and fasted rats. In the first two rows of Table I are recorded the mean values of the various fractions, in the third row the mean differences between the two groups of animals, and in the fourth row the estimates of the statistical significance of the differences, expressed in terms of  $p$  values, which indicate the percentage likelihood that such differences might arise by chance alone.

The analyses on the trichloroacetic acid extract demonstrate lower values of the inorganic and higher ones of the easily hydrolyzable P in fed rats than in fasted rats. A comparison of these data with those previously published

TABLE I  
*Mean Concentrations of Acid-Soluble P, Purine N, and Pentose in Livers of Fed and Fasting Rats*  
 The results represent the mean, in mg. per 100 gm., together with the standard error of the mean.

	No. of rats	Analyses on mercury ppt.								Analyses on trichloroacetic acid extract		
		Total organic P	Non-nucleotide P	Easily hydrolyzable P	Stable nucleotide P	Adenosine triphosphate P	Adenosine diphosphate P	Adenylic acid P	Purine N	Pentose	Inorganic P	Easily hydrolyzable P
Fed	12	50.2 ±1.1	15.0 ±1.0	19.0 ±0.7	16.1 ±0.6	13.9 ±0.8	19.6 ±0.9	1.9 ±1.2	39.8 ±0.9	102.4 ±1.4	18.8 ±0.8	21.0 ±0.8
Fasted	12	48.9 ±0.5	16.6 ±0.8	15.1 ±0.4	18.6 ±0.8	6.4 ±0.5	21.7 ±0.5	5.8 ±1.1	43.4 ±1.0	109.3 ±1.2	29.7 ±0.7	15.7 ±0.3
Difference between fed and fasted rats		+1.3 ±1.2	-1.6 ±1.3	+3.9 ±0.8	-2.5 ±1.0	+7.5 ±1.0	-2.1 ±1.0	+3.8 ±1.7	-3.7 ±1.4	-6.9 ±1.0	-10.9 ±1.0	+5.4 ±1.1
p*		0.3	0.3	<0.001	0.03	<0.001	0.05	0.04	0.02-0.01	0.001	<0.001	<0.001

\* p = the percentage chance that a deviation as great or greater than that observed would arise by chance alone; any value of p 0.05 or less is usually accepted as indicating a significant difference.

on two other series of experiments (2, 8) shows similar values for the inorganic P of both fed and fasted rats in the last two series, while the values for the easily hydrolyzable P are higher in the present than in either of the two preceding series. The statistical analysis of the data, indicating stable mean values within each of the series, suggests that individual variability is not the cause of the differences among the three series. Other factors affecting groups rather than individuals, such as preceding diet, age, season, and strain differences, may be assumed to be responsible for the discrepancies noted.

The easily hydrolyzable P in the mercury precipitate accounted for 91 to 96 per cent of the amount found in the trichloroacetic acid extract. The fact that these values did not differ from each other significantly would

TABLE II  
*Molar Distribution of Nucleotides\* in Liver of Fed and Fasting Rats*

	Total nucleotide estimated from			Adenosine triphosphate	Adenosine diphosphate	Adenylic acid	Fraction of total nucleotides present as			Phosphorylation index†
	Stable nucleotide P	Purine N	Pentose				Adenosine triphosphate	Adenosine diphosphate	Adenylic acid	
	<i>mM per kg. liver</i>	<i>mM per kg. liver</i>	<i>mM per kg. liver</i>	<i>mM per kg. liver</i>	<i>mM per kg. liver</i>	<i>mM per kg. liver</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	
Fed.....	5.2	5.7	6.8	1.5	3.2	0.6	28	61	11	2.18
Fasted.....	6.0	6.2	7.3	0.7	3.5	1.9	11	58	31	1.81

\* The values were calculated on the assumption that adenine was the only nucleotide present. Disregard of the small amount of guanylic acid occurring in liver introduces a small error into the calculations.

† Ratio of total nucleotide P to stable nucleotide P, expressing the degree of labile phosphorylation.

indicate that the easily hydrolyzable P of liver is entirely derived from nucleotides.

Values for the total organic and the non-nucleotide phosphates in the mercury precipitate were alike in fed and in fasted rats. The total nucleotide fraction was unchanged as the result of opposite shifts in the easily hydrolyzable and in the stable nucleotide phosphorus, the former of which was higher, and the latter lower, in the fed rats. The shifts in the two fractions reflected a changed mixture of nucleotides. In the fed rats adenosine triphosphate was considerably higher, adenosine diphosphate somewhat lower, and the adenylic acid greatly lower than in the fasted rats. The levels of purine nitrogen and of pentose were significantly lower in the fed rats, in agreement with the results on the stable nucleotide P.

In Table II is presented the distribution of nucleotides, calculated as

adenine compounds, in terms of molar concentration in mm per kilo of tissue. In the first three columns are recorded estimates of the molar concentration of the total nucleotides calculated from three independent measurements: (1) from the stable nucleotide phosphorus, (2) from the purine nitrogen, and (3) from the pentose value. The estimate based on the stable phosphorus does not differ significantly from the value calculated from the purine nitrogen, but the nucleotide concentration calculated from the pentose is much higher than either of the other two estimates. This difference may in part be due to the presence in the mercury precipitate of free ribose phosphate, a substance occurring in liver. Nucleotides of complex structure which contain non-purine ring systems such as alloxazine and thiamine, and therefore 2 moles of pentose per mole of purine, may also contribute to the discrepancy. According to all three estimates the concentration of total nucleotide is significantly lower in fed than in fasted rats.

In the next three columns are shown the amounts of the individual nucleotides in mm per kilo of liver, and in the following three their percentage distribution. Adenosine triphosphate accounts for about one-third of the total in fed rats and for about one-tenth in fasted rats, while the reverse is true for adenylic acid. In the last column is listed the phosphorylation index, a measure indicating the degree of labile phosphorylation of the nucleotide mixture in liver. It is expressed as the ratio of the total to the stable nucleotide phosphorus and indicates the phosphorus present in excess of that required for adenylic acid. In fed rats the phosphorylation index lies above the value required for adenosine diphosphate, while it is below this figure in fasted rats.

#### DISCUSSION

It appears worth while to consider not only the changes in the concentration, but also those in the absolute amount of the nucleotides in liver. The livers of fed rats are on the average about 60 per cent heavier than those of fasted rats, and contain therefore considerably greater absolute amounts of acid-soluble nucleotide. The rapidity of the changes in the size of the liver in the face of small variations in the total concentration of nucleotides would appear to indicate that this organ is capable of breakdown and synthesis of these compounds with great ease.

The higher level of phosphorylation in the fed rats represents a greater abundance of energy-rich bonds, probably related to the needs of the metabolism of carbohydrates in the liver. Whether the variations in the distribution of nucleotides express only changes in the energy level, or whether the concentration of the individual nucleotides in itself serves special metabolic needs, is a subject for speculation.

## SUMMARY

The distribution of acid-soluble nucleotides in the liver of rats was studied by means of a system of analyses that permitted the estimation of individual nucleotides. It was found that in fed rats adenosine triphosphate accounted for one-third, and adenylic acid for one-tenth of the nucleotides, while the reverse ratio held in fasted rats. Adenosine diphosphate accounted for about 60 per cent of the nucleotide in both groups of rats.

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# ON THE ACTIVATION OF FATTY ACID OXIDATION

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It has been previously reported from this laboratory that adenosine polyphosphates or compounds capable of phosphorylating adenylic acid are required to activate the oxidation of saturated fatty acids in crude rat liver homogenates (1). Also, Leloir and Muñoz have shown that a guinea pig liver fraction, when suitably supplemented with adenylic acid, cytochrome c, inorganic phosphate, and magnesium ions, has the power of oxidizing butyrate, *but only in the presence of some other oxidizable substrate* such as the 4-carbon dicarboxylic acids, glutamic acid, etc. (2). To explain the results of Leloir and Muñoz the author suggested that adenosine triphosphate (ATP) is an immediately necessary cofactor for the oxidation and that the function of the second oxidizable substrate and adenylic acid in the system of Leloir and Muñoz is to provide ATP by oxidative phosphorylation (1). In this paper the activation of fatty acid oxidation in easily prepared rat liver enzyme suspensions will be described. These preparations have little or no endogenous oxidative activity and are therefore much better adapted for the study of the activation than crude homogenates or the preparations of Leloir and Muñoz, which in our hands did not yield satisfactory oxidation of the higher fatty acids. It will be seen that these preparations oxidize octanoate in the presence of adenylic acid but only with the simultaneous oxidation of certain "cooxidants," confirming the data of Leloir and Muñoz. However, octanoate oxidation is completely independent of such a coupled cooxidation if ATP is supplied to the enzyme system. Previous data of the author and of Leloir and Muñoz are therefore brought into essential agreement.

The data reported here were necessary for a more detailed examination of the nature of the phosphorylative and oxidative reactions occurring in fatty acid oxidation, in particular the author's working hypothesis that a phosphorylation of the fatty acid is primary to oxidation (1).

In this paper the term cooxidant will be used to refer to the second substrate (such as fumarate,  $\alpha$ -ketoglutarate, etc.) which must be oxidized in the presence of adenylic acid to initiate or support the oxidation of the true substrate; namely, the fatty acid.

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## EXPERIMENTAL

*Preparative*—The rat liver enzyme suspensions were prepared as follows. Rat liver was removed immediately after sacrifice of the animal, chilled, cut into small pieces with scissors, and homogenized (3) in 2 parts of a cold buffer (0.0128 M sodium phosphate, pH 7.7, 0.123 M NaCl, 0.005 M KCl, 0.0012 M  $\text{MgSO}_4$ ).<sup>1</sup> The homogenate was filtered through cheese-cloth. 10 ml. of the homogenate were then centrifuged 5 minutes in a refrigerated angle head clinical centrifuge, the supernatant was discarded, and the cold buffer was added to the residue to make a total volume of 12 ml. The liver pulp was resuspended in the stoppered tube by vigorous shaking and centrifuged again for 5 minutes. The supernatant was again discarded and the resuspension in fresh buffer and centrifugation were repeated twice more. The residue after the fourth or last centrifugation was resuspended in fresh buffer to make a volume of 7 to 8 ml. The whole operation was carried out in a 15 ml. conical centrifuge tube. The suspension was used immediately (see "Requirement of DPN").

ATP, cytochrome c, diphosphopyridine nucleotide, and phosphopyruvic acid were prepared as previously mentioned (1). Adenosine diphosphate (ADP) was prepared enzymatically, with the hexokinase reaction of Colowick and Kalckar (4). Muscle adenylic acid was prepared from ATP, according to Kerr (5).  $\alpha$ -Ketoglutaric acid and oxalacetic acid were gifts of Dr. Philip P. Cohen of this department; they gave correct melting points. The fatty acids and other substrates as well as some of the inhibitors used were Eastman products and were in most cases subjected to further purification. They were presented to the enzyme as neutral sodium salts. Succinyl phosphate was prepared according to Lipmann and Tuttle (6).

*Analytical*—Acetoacetic acid was usually determined according to Greenberg and Lester (7); occasionally these values were checked manometrically with aniline citrate (8). Pentose was estimated according to Mejbaum (9),  $\alpha$ -ketoglutarate according to Friedemann and Haugen (10), and succinic acid enzymatically by the methods of Krebs (11). Oxygen uptake was determined manometrically in standard conical Warburg vessels of 15 ml. volume. The temperature was 25° and the thermal equilibration period 5 minutes; substrates were then tipped into the main compartment at zero time. Alkali and filter paper rolls were in the center well; the gas phase was air in all experiments. Wherever amounts of substrates are specified in terms of c.mm., it is assumed that 1 micromole is equivalent to 22.4 c.mm. at S.T.P.

<sup>1</sup>The composition of the medium is important in obtaining active preparations. For instance, the use of water instead of the buffer described yields totally inactive preparations.

*Activation of Octanoate Oxidation by Simultaneous Oxidation of Other Metabolites*—The enzyme suspension described is capable of oxidizing the different intermediates of the citric acid cycle in the presence of adenylic acid, magnesium ions, inorganic phosphate, and cytochrome *c*. This fact provided an opportunity for examining the activation of octanoate oxidation by the simultaneous oxidation of other metabolites, as described by Leloir and Muñoz (2). The data in Table I show that the oxidation of succinate, malate, oxalacetate, citrate, or pyruvate allowed and was necessary for the oxidation of octanoate when the suspension was supple-

TABLE I

*Activation of Octanoate Oxidation by Oxidation of Cooxidants*

The main compartment of the Warburg vessel contained 0.30 ml of enzyme suspension, 0.10 ml of adenylic acid (0.001 M),\* 0.10 ml of magnesium sulfate (0.005 M), 0.10 ml of phosphate buffer (0.0025 M), 0.10 ml of cytochrome *c* ( $1 \times 10^{-5}$  M). The side arm contained 0.20 ml of H<sub>2</sub>O or octanoate (0.007 M) and either 0.10 ml of H<sub>2</sub>O or cooxidant (0.002 M). Time, 30 minutes

Cooxidant	Octanoate	O <sub>2</sub> uptake	Acetoacetate formation
		<i>c.mms</i>	<i>c.mms</i>
None	—	—3	3
"	+	0	2
Citrate	—	22	3
"	+	63	22
Succinate	—	36	4
"	+	79	23
Malate	—	27	9
"	+	81	27
Oxalacetate	—	21	3
"	+	74	26
Pyruvate	—	37	35
"	+	58	50

\* The figures in parentheses refer to the concentration of the component in the complete reaction medium

mented with the cofactors listed above. The extra oxygen uptake in the presence of octanoate reflected actual oxidation of the fatty acids, since there was a high yield of acetoacetic acid. The conclusion may not be drawn, however, that this activation of octanoate oxidation necessarily results from any and every one-step oxidation in the citric acid cycle (such as succinate +  $\frac{1}{2}$ O<sub>2</sub> → fumarate + H<sub>2</sub>O, or malate +  $\frac{1}{2}$ O<sub>2</sub> → oxalacetate + H<sub>2</sub>O), since the enzyme suspension is capable of the nearly complete oxidation of succinic acid to CO<sub>2</sub> and H<sub>2</sub>O. The data therefore do not establish which oxidative reaction is capable of activating fatty acid oxidation, but they confirm the data of Leloir and Muñoz.

*Coupling of Octanoate Oxidation with Reaction,  $\alpha$ -Ketoglutarate +  $\frac{1}{2}O_2 \rightarrow$  Succinate +  $CO_2$  +  $H_2O$* —Although sodium malonate inhibits fatty acid oxidation somewhat in the preparations described, it was found possible to choose a concentration of malonate which completely blocked succinate oxidation but still allowed considerable octanoate oxidation. In the presence of 0.01 M malonate the oxidation of 0.002 M succinate was inhibited completely. When octanoate was present in such a system, it was not oxidized. When 0.002 M  $\alpha$ -ketoglutarate was substituted for the succinate it was oxidized completely to succinate, as shown by oxygen uptake data and analytical data on both  $\alpha$ -ketoglutarate and succinate (Table II; Fig. 1). However, in the latter situation the oxidation of  $\alpha$ -ketoglutarate allowed octanoate oxidation. Therefore octanoate oxidation can be coupled to a single, well defined cooxidation. The data of Leloir and

TABLE II

*Balance Sheet for Activation of Octanoate Oxidation by  $\alpha$ -Ketoglutarate Oxidation*

The experimental details are exactly as in Fig. 1; all volumes are tripled to allow analytical studies. Time, 40 minutes.

Oxidation No.	Substrate	Cooxidant	O <sub>2</sub> uptake	$\alpha$ -Ketoglutarate disappeared	Succinate formed	Acetoacetate formed
			c.mm.	c.mm.	c.mm.	c.mm.
1	None	$\alpha$ -Ketoglutarate (259 c.mm.)	133	248	260	32
2	Octanoate	" (259 " )	438	232	278	240
3	None	Succinate (269 c.mm.)	10			8
4	Octanoate	" (269 " )	3			9
5	None	None	17			16
6	Octanoate	"	12			24

Muñoz focused attention on the oxidation of the 4-carbon dicarboxylic acids as being active in the initiation of fatty acid oxidation; the data reported here show that other oxidative reactions may function in this activation.

Another point of importance is that when the  $\alpha$ -ketoglutarate was completely oxidized, there was no further oxidation of octanoate. It would appear from this fact that the rate-limiting reaction during octanoate oxidation is probably the activating reaction, which depends on the activating oxidation. Otherwise one might expect a continued extra O<sub>2</sub> uptake (due to oxidation of octanoate intermediates) after  $\alpha$ -ketoglutarate oxidation is complete. In experiments comparable to those described, the introduction of more  $\alpha$ -ketoglutarate from a second side arm when the plateau was reached resulted in reestablishment of octanoate oxidation.

The oxidation of octanoate in the presence of 0.01 M malonate also

appears to rule out a possible function of the 4-carbon dicarboxylic acids as necessary hydrogen-transport factors in fatty acid oxidation.

*Activation of Octanoate Oxidation by ATP*—Earlier work showed that ATP was required for octanoate oxidation in liver homogenates (1). The suggestion made in that paper that the oxidation of cooxidants was necessary for the formation of ATP by oxidative phosphorylation of adenylic acid was supported by the fact that  $\alpha$ -ketoglutarate oxidation is capable

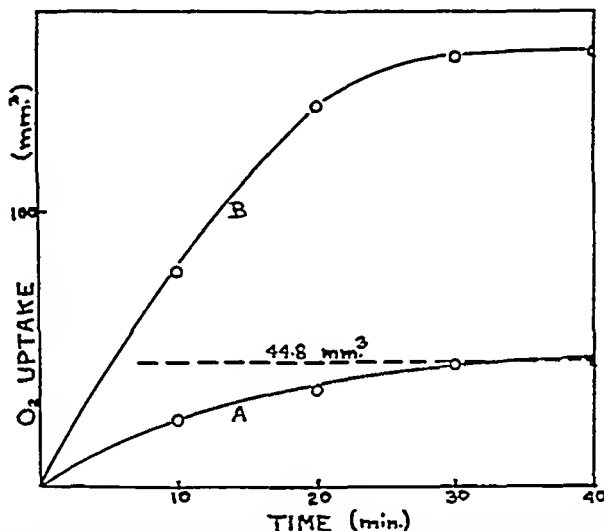


FIG. 1. The coupling of octanoate oxidation with  $\alpha$ -ketoglutarate oxidation. The main compartment contained 0.60 ml. of enzyme, 0.20 ml. of adenylic acid (0.001 M), 0.20 ml. of  $\text{MgSO}_4$  (0.005 M), 0.20 ml. of phosphate buffer (0.0025 M), 0.20 ml. of cytochrome c ( $1 \times 10^{-3}$  M), 0.20 ml. of sodium malonate (0.01 M). The side arm contained 0.20 ml. of  $\alpha$ -ketoglutarate (0.002 M) and 0.40 ml. of  $\text{H}_2\text{O}$  or octanoate (0.007 M). Curve A shows the oxidation of  $\alpha$ -ketoglutarate alone proceeding to the calculated 44.8 c.mm. of  $\text{O}_2$  uptake for the amount added and Curve B shows the oxygen uptake for the two substrates together.

of activating fatty acid oxidation, since Ochoa has shown that the reaction,  $\alpha$ -ketoglutarate +  $\frac{1}{2}\text{O}_2 \rightarrow$  succinate +  $\text{CO}_2$  +  $\text{H}_2\text{O}$ , is capable of vigorous esterification of inorganic phosphate (12). A direct trial of ATP, substituted for adenylic acid +  $\alpha$ -ketoglutarate, was then made, all other factors remaining constant. Surprisingly, there was no activation of octanoate oxidation (see Table III), even in the presence of 0.003 M ATP, a concentration higher than that necessary for maximal activation in the liver homogenate previously described (1). This finding was at direct

variance with the known activation of octanoate oxidation by ATP in the crude homogenate.

On careful study of the reaction conditions and the concentrations of the different components, it was found that the concentration of octanoate was critical in limiting activation by ATP. Octanoate was present usually in a concentration of 0.007 M; ATP was not capable of activating oxidation in these preparations at this concentration of octanoate. However, when octanoate was present at 0.001 M concentration, ATP activated its oxidation. The data in Table IV show the essential facts.  $\alpha$ -Ketoglutarate oxidation is capable of activating the oxidation of octanoate at any concentration tested, although not maximal at 0.01 M octanoate. On the

TABLE III

*Failure of Adenosine Triphosphate to Substitute for  $\alpha$ -Ketoglutarate Plus Adenylic Acid in Oxidation of 0.007 M Octanoate*

The main compartment contained 0.30 ml. of enzyme, 0.10 ml. of adenylic acid (0.001 M) or ATP, 0.10 ml. of  $\text{MgSO}_4$  (0.005 M), 0.10 ml. of cytochrome c ( $1 \times 10^{-5}$  M), 0.10 ml. of sodium malonate (0.01 M), 0.10 ml. of phosphate buffer. The side arm contained 0.10 ml. of  $\alpha$ -ketoglutarate (0.002 M) or  $\text{H}_2\text{O}$ , and 0.20 ml. of octanoate (0.007 M) or  $\text{H}_2\text{O}$ . Time, 30 minutes.

Activator	Octanoate	O <sub>2</sub> uptake	Acetoacetic acid formation
		c.mm.	c.mm.
$\alpha$ -Ketoglutarate + adenylic acid.....	—	22	6
“ + “ “ .....	+	79	39
ATP (0.001 M).....	—	0	2
“ (0.001 “).....	+	—4	4
“ (0.002 “).....	—	0	2
“ (0.002 “).....	+	2	7
“ (0.003 “).....	—	0	2
“ (0.003 “).....	+	6	6

other hand, ATP is only able to activate maximally at octanoate concentrations of 0.001 M. It would appear at first glance that two mechanisms for the activation exist. However, the peculiar effect of fatty acid salts and other surface active compounds in inhibiting enzymes has already been pointed out (1). Recently, Boyer has demonstrated some peculiar effects of octanoate in protecting serum albumin against denaturation (13). In view of this surface activity of octanoate toward proteins, it would not necessarily appear justified at present to assume two different activating mechanisms, since it is possible that the ATP formed during  $\alpha$ -ketoglutarate oxidation is generated on or near the activating enzyme surface, and therefore not necessarily liable to the inhibition by octanoate. Fairbairn has recently shown that the concentration of free fatty acids

in normal tissues is extremely small, probably lower than 0.001 M (14). It is also known (15) that the concentration of adenosine polyphosphates in normal rat liver is relatively great, approximately 0.002 M.

The rate of octanoate oxidation depends on the concentration of ATP. In the presence of 0.001 M ATP the oxidation falls off rapidly and stops in 10 to 15 minutes. If more ATP is added from a second side arm, the oxidation is reestablished. Usually 0.002 M ATP keeps the reaction going for 20 to 30 minutes.

Malonate was included in all experiments because it inhibited effectively the endogenous oxygen uptake of the enzyme.

TABLE IV

*Effect of Octanoate Concentration on Activation by Adenosine Triphosphate*

The details are exactly as given in Table III. Time, 25 minutes. ATP concentration, 0.0024 M.

Activator system	Octanoate	Octanoate concentration	O <sub>2</sub> uptake	Acetoacetate formation
		M	c mm	c mm
$\alpha$ -Ketoglutarate + adenylic acid	—		20	6
“ “ “ “	+	0.01	69	37
ATP . . . . .	—		2	2
“ . . . . .	+	0.01	—6	2
$\alpha$ -Ketoglutarate + adenylic acid	—		20	6
“ “ “ “	+	0.005	94	47
ATP . . . . .	—		2	2
“ . . . . .	+	0.005	12	7
$\alpha$ -Ketoglutarate + adenylic acid	—		20	6
“ “ “ “	+	0.001	89	52
ATP . . . . .	—		2	2
“ . . . . .	+	0.001	69	46

*Specific Activation by ATP*—In crude liver homogenates both ATP and ADP activated octanoate oxidation equally well (1). When the adenine nucleotides were tested (Table V) for their ability to activate octanoate oxidation in the enzyme suspension used here (the octanoate concentration being 0.001 M), only ATP was found to be active. The slight activation by ADP was possibly due to traces of ATP in the ADP. Both ADP and adenylic acid were active when  $\alpha$ -ketoglutarate was added as the cooxidant. Phosphopyruvic acid, sodium pyrophosphate, diphosphopyridine nucleotide, and thiamine pyrophosphate were unable to activate the oxidation. ATP is therefore a specific activator for the oxidation.

*Adenylic Acid Requirement during Activation by Oxidation of Cooxidants*—



When the requirement of the different components for oxidation of octanoate (activated by  $\alpha$ -ketoglutarate oxidation in the presence of malonate) was studied (Table VI), it was found that adenylic acid could be omitted from the system without completely impairing the rate of octanoate oxidation. This was unexpected, since all experimental data presented previously on the activation have emphasized the necessity for the presence of the adenine nucleotides. Furthermore, Ochoa has shown that adenine nucleotide is required for the oxidation of  $\alpha$ -ketoglutarate (12). The

TABLE V

*Specificity of Adenosine Triphosphate for Activation*

The main compartment contained 0.30 ml. of enzyme, 0.10 ml. of cytochrome *c* ( $1 \times 10^{-5}$  M), 0.10 ml. of malonate (0.01 M), 0.10 ml. of  $\text{MgSO}_4$  (0.005 M), 0.10 ml. of buffer (0.0025 M), 0.10 ml. of adenine nucleotides or other phosphate esters (0.0025 M). The side arm contained 0.10 ml. of  $\text{H}_2\text{O}$  or  $\alpha$ -ketoglutarate (0.002 M), and 0.20 ml. of octanoate (0.001 M) or  $\text{H}_2\text{O}$ . Time, 25 minutes.

Activator	Octanoate	O <sub>2</sub> uptake	Acetoacetic acid formation
		<i>c.mm.</i>	<i>c.mm.</i>
ATP.....	—	2	2
	+	58	36
Adenosine diphosphate.....	—	2	2
	+	11	7
Adenylic acid.....	—	2	2
	+	-2	3
ADP + $\alpha$ -ketoglutarate.....	—	16	7
	+	72	42
Adenylic acid + $\alpha$ -ketoglutarate.....	—	16	10
	+	76	44
Phosphopyruvic acid.....	—	6	4
	+	-2	3
Thiamine pyrophosphate.....	—	4	4
	+	5	4
Sodium ".....	—	2	4
	+	0	2

absence of adenine nucleotide reduced the rate of  $\alpha$ -ketoglutarate oxidation greatly, as expected, but the reduction of octanoate oxidation was not proportional. This was true in many experiments. To determine whether this effect was true for other cooxidants as well, the activation of octanoate oxidation by a series of other cooxidants was measured with and without added adenylic acid (Table VII). It was found that  $\alpha$ -ketoglutarate was the only cooxidant which activated octanoate oxidation *without* added adenylic acid. Oxalacetate gave a slight activation in some experiments. It is possible that the requirement of adenine nucleotide

for  $\alpha$ -ketoglutarate oxidation is smaller than for other oxidative reactions requiring adenylic acid, and that this requirement is met by adenine nucleotide already present in the enzyme suspension. Trichloroacetic acid filtrates of the enzyme (without added adenine nucleotide) showed the presence of considerable pentose which, if calculated on the basis that all of it was adenylic acid pentose, would represent about  $3 \times 10^{-4}$  M adenylic acid in the unsupplemented enzyme mixture. This pentose was for the most part tenaciously retained even after thorough dialysis.

It has been shown already that  $\alpha$ -ketoglutarate oxidation is capable of activating octanoate oxidation under circumstances in which ATP is not capable of activation (*i.e.* with high octanoate concentration). The finding that  $\alpha$ -ketoglutarate oxidation is capable of activation *without*

TABLE VI  
*Components of  $\alpha$ -Ketoglutarate Coupled System*

The experimental details are exactly as given in Fig. 1. H<sub>2</sub>O is substituted for the component removed. Time, 40 minutes.

	Octanoate	O <sub>2</sub> uptake	Acetoacetic acid formation
		<i>c mm.</i>	<i>c mm.</i>
Complete system	—	45	5
“ “	+	146	80
No $\alpha$ -ketoglutarate	—	-3	0
“ “	+	-4	+2
No Mg ++	—	23	5
“ “ “	+	60	18
No cytochrome c	—	39	7
“ “	+	131	71
No adenylic acid	—	18	4
“ “ “	+	90	32

added adenine nucleotide would appear to be supporting evidence that octanoate oxidation can be activated by two different mechanisms, one by ATP and the other through the oxidation of  $\alpha$ -ketoglutarate, but not necessarily involving the intermediate formation of ATP by oxidative phosphorylation. It has been presumed that a phosphorylated intermediate of  $\alpha$ -ketoglutarate arises during  $\alpha$ -ketoglutarate oxidation by uptake of inorganic phosphate (to account for the esterification of inorganic phosphate during this oxidation) (12). By analogy with pyruvate oxidation in certain bacteria, in which acetyl phosphate has been established as an intermediate by Lipmann (16), Ochoa has brought up the possibility that succinyl monophosphate may be the phosphorylated intermediate formed during  $\alpha$ -ketoglutarate oxidation (12). To test the question

whether succinyl monophosphate is the phosphorylated intermediate arising during  $\alpha$ -ketoglutarate oxidation which is capable of activating fatty acid oxidation, synthetic succinyl phosphate, a mixture of the mono- and diphosphates (6), was substituted for  $\alpha$ -ketoglutarate in the co-oxidation experiments described above. This obviously impure material was not capable of substituting for  $\alpha$ -ketoglutarate oxidation, with or without added adenylic acid.

The possibility must be left open that some phosphorylated intermediate arises during  $\alpha$ -ketoglutarate oxidation which may be capable of phosphorylating or activating the fatty acid and which does not necessarily act through the adenylic acid system.

TABLE VII

*Activation of Octanoate Oxidation by Oxidation of Cooxidants in Presence and Absence of Adenylic Acid*

The conditions are exactly as given in Fig. 1. The cooxidants are present in the final concentration of 0.002 M. Time, 28 minutes.

Cooxidant	Octanoate	O <sub>2</sub> uptake	
		With 0.001 M adenylic acid	No adenylic acid
		<i>c.mm.</i>	<i>c.mm.</i>
$\alpha$ -Ketoglutarate.....	—	44	19
“ .....	+	160	90
Fumarate.....	—	37	10
“ .....	+	120	14
Oxalacetate.....	—	21	4
“ .....	+	140	13
Succinate.....	—	42	19
“ .....	+	111	19
Citrate.....	—	20	10
“ .....	+	71	2

*Oxidation of Normal Saturated Fatty Acids of Varying Chain Length—*

It was previously shown that rat liver homogenates can oxidize all the normal saturated fatty acids having from 2 to 18 carbon atoms (1). The washed enzyme suspension described here was capable of oxidizing fatty acids having more than 4 carbon atoms in the presence of ATP. Acetate was not attacked. The rate of oxidation increased with an increase in the length of the carbon chain. Representative data are shown in Table VIII.

*Sensitivity to Sulfhydryl Reagents—*The oxidation of octanoate (activation by ATP) is sensitive to certain sulfhydryl reagents. The following reagents, known to inhibit more or less selectively many enzymes requiring

a free sulfhydryl group for activity, inhibited the oxidation: 0.001 M iodoacetate, 10 per cent; 0.001 M iodoacetamide, 40 per cent;  $5 \times 10^{-4}$  M *p*-chloromercurobenzoate, 50 per cent;  $5 \times 10^{-4}$  M potassium ferricyanide, 100 per cent;  $5 \times 10^{-4}$  M *o*-iodosobenzoic acid, 100 per cent;  $1 \times 10^{-4}$  M iodine, 95 per cent. It therefore appears that one or more of the enzymes

TABLE VIII

*Oxidation of Different Fatty Acids*

The main compartment contained 0.60 ml. of enzyme, 0.20 ml. of cytochrome *c* ( $2 \times 10^{-5}$  M), 0.20 ml. of  $\text{MgSO}_4$  (0.005 M), 0.20 ml. of malonate (0.01 M), 0.20 ml. of buffer, 0.20 ml. of adenosine triphosphate (0.0025 M). The side arm contained 0.40 ml. of fatty acid (0.001 M) or  $\text{H}_2\text{O}$ . Time, 20 minutes.

Oxidation No	Substrate	O <sub>2</sub> uptake	Acetoacetic acid formation
		<i>c mm</i>	<i>c mm</i>
1	None	2	6
2	Acetate	3	8
3	Butyrate	17	17
4	Octanoate	78	56
5	Palmitate	112	68

TABLE IX

*Fatty Acid Esters As Substrates*

Exactly as given in Table VIII. The esters were finely dispersed in water in such concentration as to yield 0.001 M potential fatty acid concentration. Tween 40, 2.5 per cent aqueous solution. Time, 21 minutes.

Substrate	O <sub>2</sub> uptake	Acetoacetate formation
	<i>c mm</i>	<i>c mm</i>
None ..	0	2
Sodium octanoate	83	54
Ethyl "	28	17
Glyceryl trioctanoate	0	3
Tween 40	2	2
Glyceryl trioctanoate + sodium octanoate	70	38

of the activation-oxidation complex requires free sulfhydryl groups for activity.

*Necessity for Free Carboxyl Group in Fatty Acid for Activation by ATP*—To determine whether a free carboxyl group is required for ATP activation, three fatty acid esters were substituted for octanoate and the rates of oxidation determined. Ethyl octanoate, glyceryl trioctanoate, and Tween 40 were tested as substrates (Table IX). The latter compound is a mono-

palmityl ester of polyoxyalkylene sorbitol anhydride, a product of the Atlas Powder Company. It is water-soluble and has been successfully used as a lipase substrate by Gomori (17). Of these three compounds only ethyl octanoate was attacked, at a rate of one-third of that of sodium octanoate. On testing lipase and esterase activities of the enzyme suspension, it was found that only ethyl octanoate was attacked, the other esters remaining unhydrolyzed. The positive oxidation of the ethyl ester must therefore be attributed to the oxidation of the octanoic acid liberated by the esterase. The insolubility or surface activity of glyceryl trioctanoate did not appear to be a factor in its failure to be oxidized; when sodium octanoate was added along with glyceryl trioctanoate, its oxidation was inhibited only 10 or 15 per cent by the presence of the insoluble triglyceride.

TABLE X

*Requirement of Diphosphopyridine Nucleotide in Aged Enzyme Preparations*

The details are given as in Table IX, with the addition of 0.10 ml. of H<sub>2</sub>O or DPN ( $5 \times 10^{-4}$  M) in the side arm. Time, 35 minutes. Octanoate (0.001 M) was substrate.

Enzyme	Octanoate	DPN	O <sub>2</sub> uptake c.mm.
Fresh.....	—	—	2
" .....	+	—	98
" .....	—	+	4
" .....	+	+	105
Aged 3 hrs. at 0°.....	—	—	0
" 3 " " 0°.....	+	—	4
" 3 " " 0°.....	—	+	4
" 3 " " 0°.....	+	+	110

In comparable experiments *n*-octyl alcohol and *n*-octaldehyde were not oxidized with or without the presence of ATP. It must be concluded from experiments of this type that a free carboxyl group is necessary for the activation of fatty acid oxidation by ATP.

*Requirement of DPN*—When the preparations described were aged at 0° for 3 or 4 hours, they were no longer active with the usual supplements. When diphosphopyridine nucleotide was added, these preparations regained nearly all of the lost activity. Diphosphopyridine nucleotide (DPN) therefore appears to be involved in the oxidation mechanism. Dialysis for 3 hours with continuous stirring resulted in inactivation which was *not* restored by DPN with or without the simultaneous addition of thiamine pyrophosphate and a crude preparation of flavin adenine nucleotide (about 5 per cent pure). This inactivation was also brought about by aging without dialysis for more than 4 hours. It could be retarded

by the addition of adenylic acid to the otherwise unsupplemented enzyme. The protective effect of adenylic acid was very pronounced. It may be related to the fact that considerable bound pentose is present in the preparation even after dialysis, and that the suspension contains a very active 5-nucleotidase which splits inorganic phosphate from added adenylic acid. It is possible that some essential component of the enzyme system possesses a conjugated protein structure which is either readily denatured or inactivated by enzymatic action of phosphatases, etc.

It is clear from the data in Table X that diphosphopyridine nucleotide must be added to the list of cofactors concerned in fatty acid oxidation, a list which is evidently not yet complete. The DPN used in these experiments was about 40 per cent pure and was free of triphosphopyridine nucleotide.

#### DISCUSSION

It must be concluded from this investigation that the activation of fatty acid oxidation by ATP and the activation by the cooxidation of other substrates described by Leloir and Muñoz are essentially the same process, ATP being the immediately necessary factor for the activation. Many investigators, including Leloir and Muñoz, have demonstrated that oxidations in the so called citric acid cycle are capable of esterifying inorganic phosphate. The mechanism of such esterification is not known, except in the case of glyceraldehyde phosphate oxidation (18) and in pyruvate oxidation in certain bacteria (16). However, it is believed that such oxidative esterification universally leads to the formation of ATP (since adenylic acid appears to be necessary in such oxidations), and that recovery of other phosphorylated intermediates as a result of such oxidations (*i.e.* phosphocreatine, hexose phosphates) is a reflection of the presence of the corresponding transphosphorylating enzymes. The enzyme suspensions used in this investigation did not in general show an accumulation of acid-labile phosphate during fumarate oxidation. The high phosphatase activity of the suspensions was probably responsible. The addition of yeast hexokinase and glucose to such a system, however, allowed active phosphorylation.

The washed enzyme suspension used here represents a considerable concentration of the fatty acid oxidase system. The  $\Delta Q_{O_2}$  (c.mm. of oxygen taken up in the presence of octanoate above the oxygen uptake of the tissue without substrate per mg. of dry weight per hour) of these preparations ranges between 25 and 35. This figure for liver slices ranges between 4 and 10 (19).

The data presented here have been used as a starting point for a detailed examination of the mechanism of activation by ATP. The author's

working hypothesis, namely that ATP activates the oxidation by a preliminary phosphorylation of the fatty acid (1), will be considered in detail elsewhere.

Another problem which has been approached experimentally with this enzyme suspension is the nature of the 2-carbon fragments, derived from the long chain fatty acids by oxidation, which undergo condensation to form the ketone bodies (20). This work will also be reported in detail.

It is also timely to point out that the enzyme system described here is very likely the same system described by Leloir and Muñoz and must be considered as representing the physiological oxidation of the fatty acids to ketone bodies (probably lacking, however, in physiological control mechanisms). This point is made because a number of fatty acid dehydrogenases have been described (21-23) which dehydrogenate saturated fatty acids anaerobically in the presence of adenylic acid or certain purines, yielding in at least one case (22) unsaturated acids, with the unsaturation near the middle of the fatty acid carbon chain. The author considers the latter group of enzymes to be concerned primarily with the physiological regulation of the degree of saturation of the tissue lipids and not necessarily concerned with the energy-yielding oxidation of the fatty acids to carbon dioxide and water through the stage of the ketone bodies. The two groups of enzymes differ both in properties and distribution. For instance, the enzyme suspension used here is unable to dehydrogenate long chain fatty acids in the presence of adenylic acid with the Thunberg technique, but it is capable of this dehydrogenation if ATP is present. On the other hand the second class of dehydrogenases does not require ATP but requires either adenylic acid (21, 22) or xanthine (23) as a coenzyme.

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#### SUMMARY

1. A washed rat liver suspension, supplemented with adenylic acid, cytochrome c, magnesium ions, and inorganic phosphate, is capable of oxidizing saturated normal fatty acids with the production of acetoacetic acid, but *only* with the simultaneous oxidation of some other oxidizable metabolite.

2. The oxidation of the fatty acid can be coupled to a specific one-step cooxidation, that of  $\alpha$ -ketoglutarate to succinate.

3. The oxidation of fatty acids is completely independent of such coupled oxidations if adenosine triphosphate is present. This fact leads to the

conclusion that the coupled oxidation of other substrates activates fatty acid oxidation by providing ATP during the oxidative phosphorylation of adenylic acid.

4. ATP is specific for the activation; adenosine diphosphate (ADP), adenylic acid, and other phosphate esters are inactive.

5. The activation-oxidation complex is sensitive to sulfhydryl reagents.

6. A free carboxyl group is necessary for the activation; fatty acid esters are not attacked unless a lipase or esterase is present.

7. Diphosphopyridine nucleotide is an essential component of the fatty acid oxidase system.

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# EFFECT OF NICOTINIC ACID AMIDE AND SODIUM ON GLYCOLYSIS AND OXYGEN UPTAKE IN BRAIN HOMOGENATES\*

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The glycolytic activity of brain tissue is rapidly lost on destruction of its cell structure. Geiger (1) found that when brain cells are disrupted with distilled water an inhibitor of glycolysis is released. By simply diluting a water extract of rat brain, he reduced the effect of the inhibitor and obtained a rate of lactic acid production from glucose by far exceeding the values obtained with brain slices or brain dispersions. These observations were confirmed by Ochoa (2).

We attempted to obtain similar active extracts from mouse brain but were unsuccessful. Extracts prepared by Geiger's method were not only inactive, but markedly inhibitory when added to an actively glycolyzing rat brain extract. It was decided to study the mechanism of this inhibition in the hope of finding means to eliminate the inhibitory factors and to obtain actively glycolyzing brain preparations.

Evidence has been obtained that the inhibition in brain homogenates is caused by two components. One inhibitor affects the utilization of triose phosphate; the second inhibits the phosphorylation of glucose.

Many tissues contain an enzyme which inactivates diphosphopyridine nucleotide (DPN). Mann and Quastel (3) have shown that the destruction of DPN can be prevented by the addition of nicotinic acid amide. When DPN and nicotinic acid amide were added to mouse brain homogenates in the presence of a phosphate acceptor, rapid utilization of hexose diphosphate took place.

The breakdown of glucose, however, was found to be markedly affected by the presence of  $\text{Na}^+$ . Elimination of  $\text{Na}^+$  from the added reagents and addition of adenosine triphosphate (ATP), DPN, and nicotinic acid amide resulted in a rapid production of lactic acid from glucose.

## EXPERIMENTAL

### *Methods*

Swiss albino mice (CFW strain, Carworth Farms) were used. The animals were killed by decapitation, and the brains removed immediately.

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Homogenates were made by grinding the brain thoroughly in a mortar and then diluting the suspension with 10 volumes of distilled water, which was added in small portions. Glass-distilled water was used throughout.

Lactic acid was determined by the method of Barker and Summerson (4). Glucose was estimated according to the procedure of Shaffer and Somogyi (5) with a protein-free filtrate prepared according to West, Scharles, and Peterson (6). The acid-soluble phosphate fractions were determined after conversion to orthophosphate by the methods developed by Meyerhof and his school (7).

Another method of following the utilization of glucose or hexose phosphates is based on the decolorization of methylene blue in Thunberg tubes. Glucose and the hexose phosphate esters are broken down to triose phosphate by the brain homogenate, which then decolorizes methylene blue through the triose phosphate dehydrogenase enzyme system. For rapid preliminary determinations of the optimal activity of the brain homogenates under various conditions this method was found to be very useful. For determinations of oxygen uptake the conventional manometric methods with the Warburg-Barcroft apparatus were employed.

### *Materials*

DPN of 55 to 70 per cent purity was prepared from bakers' yeast (Fleischmann) by the method of Williamson and Green (8), as modified by Ochoa.<sup>1</sup> The barium salt of ATP was prepared from rabbit muscle essentially according to the procedure of Lohmann (9). The calcium salt of phosphocreatine, freed from sodium by reprecipitation from alcohol, was synthesized according to the method of Zeile and Fawaz (10). The calcium salt of hexose diphosphate was a commercial product obtained from the Schwarz Laboratories, Inc., New York. All reagents were converted into the potassium or ammonium salts and brought to a pH of 7.4 with KOH and HCl before use.

### *Results*

*Glycolysis by Extracts of Mouse Brain*—It can be seen from the data in Table I that in mouse brain homogenates prepared with saline, about 400  $\gamma$  of lactic acid are formed from glucose per 100 mg. of wet weight of brain per hour. When distilled water is used instead of saline, very small amounts of lactic acid are formed. A diluted brain extract prepared according to Geiger (1) from young rats actively utilizes glucose anaerobically, while a similar extract from mouse brain is inactive. Moreover, such an extract of mouse brain inhibits completely an actively glycolyzing rat brain extract.

<sup>1</sup> Ochoa, S., personal communication.

*Metabolism in Brain Homogenates*—To investigate further the effect of distilled water on brain metabolism, activities of several brain dehydrogenases were examined by the Thunberg method in which saline homogenates were compared with distilled water preparations. It was apparent that all dehydrogenases which require DPN were markedly inhibited in the distilled water preparations, while the succinic acid dehydrogenase, which does not require DPN, was not significantly affected. The results of a typical experiment are shown in Table II.

An enzyme which destroys DPN (referred to here as DPNase) has been shown to occur in many tissues. Mann and Quastel (3) demonstrated

TABLE I  
*Inhibition of Glycolysis by Extracts of Mouse Brain*

The brain preparation was made according to the method of Geiger with 4 volumes of distilled water. 0.2 ml. of extract was used in each Warburg vessel. The final concentrations of the solutions in a 2 ml. volume were glucose, 0.016 M; NaHCO<sub>3</sub>, 0.016 M; MgCl<sub>2</sub>, 0.0035 M; sodium phosphate-potassium phosphate buffer, pH 7.3, 0.01 M; DPN (diphosphopyridine nucleotide), 0.0004 M; ATP (adenosine triphosphate), 0.001 M. Incubated for 1 hour at 37° in the Warburg-Barcroft apparatus with nitrogen containing 5 per cent CO<sub>2</sub> as the gas phase.

Experiment No.	Brain preparation	Additions*	Lactic acid per 100 mg. wet weight of brain
1	Mouse brain, saline homogenate	Glucose, NaHCO <sub>3</sub>	400
2	Mouse brain water extract (Geiger)	“ “ MgCl <sub>2</sub> , phosphate, ATP, DPN	100
3	Rat brain, water extract	Same as Experiment 2	1275
4	Same as Experiment 3 + 0.2 ml. mouse brain extract	“ “ “ 2	180

\* Glucose was tipped in from the side bulb at the beginning of the experiment.

that this enzyme is inhibited by nicotinic acid amide which counteracts the destruction of DPN. The effect of adding DPN and nicotinic acid amide to distilled water and saline homogenates of brain is recorded in Table III.

The results shown in Table III suggest that in brain homogenates prepared with distilled water DPN is much more rapidly inactivated than when saline is used. This may well be due to the cytolytic effect of distilled water and to the release of DPNase into solution.

The inhibition of DPNase is a function of the concentration of nicotinic acid amide. While low concentrations were ineffective in preventing the

TABLE II

*Comparison of Dehydrogenase Activities in Saline and Water Homogenates of Mouse Brain*

The brain preparation was made by grinding and diluting with 10 volumes of distilled water or saline. 0.5 ml. of homogenate was used for each experiment. The final concentrations of the solutions in a 3 ml. volume were sodium phosphate buffer, pH 7.3, 0.02 M; methylene blue, 0.00013 M, tipped in from the side bulb at the beginning of the experiment. Incubated at 37° in evacuated Thunberg tubes. End-point, complete decolorization.

Experiment No.	Mouse brain homogenate	Substrate in molar concentration	Reduction time
			min.
1	Saline	Lactate, 0.025	6.5
2	"	Succinate, 0.02	8.5
3	"	Pyruvate, 0.018	7.0
4	"	Glutamate, 0.01	12.0
5	"	"	28.0
6	Water	Lactate, 0.025	109.0
7	"	Succinate, 0.02	12.0
8	"	Pyruvate, 0.018	108.0
9	"	Glutamate, 0.01	>180.0
10	"	"	>300.0

TABLE III

*Effect of Nicotinic Acid Amide on Dehydrogenase Activities of Brain Homogenates*

The experimental conditions were as in Table II. The Thunberg tubes were incubated for 10 minutes at room temperature after nicotinic acid amide (NAA) 0.0095 M, and diphosphopyridine nucleotide (DPN), 0.0002 M, were added to the brain homogenates. Methylene blue, 0.00026 M, was tipped in from the side bulb of the evacuated Thunberg tubes at the beginning of the experiment.

Experiment No.	Mouse brain homogenate	Additions		Substrate* in molar concentration	Reduction time
		DPN	NAA		
					min.
1	Saline	+	-	Lactate, 0.02	13.0
2	"	+	+	" 0.02	7.0
3	"	+	-	Glucose, 0.016	10.0
4	"	+	+	" 0.016	8.5
5	Water	+	-	Lactate, 0.02	108.0
6	"	+	+	" 0.02	8.0
7	"	+	-	Glucose, 0.016	47.0
8	"	+	+	" 0.016	8.0
9	"	+	-	Hexose diphosphate, 0.01	47.0
10	"	+	+	" "	4.5

\* When glucose or hexose diphosphate was used as substrate, 0.001 M adenosine triphosphate was added to act as the phosphate-transferring agent.

destruction of DPN, higher concentrations were found to be inhibitory for the dehydrogenase systems tested. An optimal range of nicotinic acid amide concentration had to be established with the experimental conditions used, since changes in its concentration considerably alter the effect of phosphate buffer on reduction time, as shown in Table IV.

When glucose was used as substrate for brain homogenates to which suitable amounts of ATP, DPN, and nicotinic acid amide were added, it was found to be utilized only half as rapidly as hexose diphosphate. This pointed to the phosphorylation of hexose as the pace-maker of the reactions,

TABLE IV

*Effect of Phosphate Concentration on Destruction of Diphosphopyridine Nucleotide in Presence of Nicotinic Acid Amide*

The experimental conditions were as in Table III. Substrate, sodium lactate, 0.025 M.

Experiment No.	Additions		Reduction time
	Phosphate buffer	Nicotinic acid amide	
	M	mg.	min.
1	0.02	0.2	51.0
	0.0033	0.2	122.0
2	0.02	0.8	11.0
	0.0033	0.8	33.0
3	0.02	4.0	9.0
	0.0033	4.0	11.0
4	0.02	32.0	11.0
	0.0033	32.0	14.0
5	0.02	100.0	74.0
	0.0033	100.0	49.0
6	0.02		116.0
	0.0033		150.0

either because of a low concentration of the enzymes required or due to other factors involved in phosphate transfer.

*Effect of Sodium on Glycolysis*—Potassium and sodium ions have been reported to influence phosphorylations (11, 12). When potassium or ammonium buffers were used instead of sodium salts and all solutions neutralized with KOH, a striking increase in glycolytic activity of brain homogenates occurred.

With glucose as substrate, addition of 0.04 M NaCl produced a marked inhibition which was absent when hexose diphosphate was used as substrate. To investigate further the mechanism of the sodium inhibition a balance sheet was drawn up for glucose disappearance, lactic acid production, and the acid-soluble phosphate fractions (Table V).

It became apparent that not only was lactic acid production affected by  $\text{Na}^+$ , but also hexose phosphate esters failed to appear. The values for the P fraction obtained by subtracting the P after 7 minutes hydrolysis in  $\text{N HCl}$  from the total acid-soluble P represent chiefly the hexose phosphate and phosphoglyceric acid which are formed during glycolysis as well as 1 P atom in ATP and the phosphorus in DPN which were added to the glycolyzing system. On correction for the two latter compounds, it was found that no phosphate esters are formed in the presence of added  $\text{Na}^+$ . Negligible amounts of glucose disappear.

TABLE V

*Effect of Sodium and Phosphocreatine on Glycolysis by Brain Homogenates*

The brain preparation was made by grinding and diluting with 10 volumes of distilled water. 0.3 ml. of homogenate was used per experiment. The final concentrations of the solutions in a 2 ml. volume were ammonium phosphate buffer, pH 7.3, 0.005 M;  $\text{KHCO}_3$ , 0.008 M;  $\text{MgCl}_2$ , 0.0035 M; ATP (adenosine triphosphate), 0.001 M; DPN (diphosphopyridine nucleotide), 0.0004 M; nicotinic acid amide, 0.012 M; glucose, 0.012 M; DPN was tipped in from the side bulb at the beginning of the experiment. Incubation at  $37^\circ$  for 1 hour in evacuated Thunberg tubes.

Experiment No.	Additions		Change after 1 hr.			
	Phospho-creatine	NaCl	ATP P*	Ester P*	Glucose	Lactic acid
	M	M	micromoles	micromoles	micromoles	micromoles
1			0.0	+2.9	-11.3	+21.1
2	0.0055		+1.77	+6.7	-15.3	+24.9
3		0.04	-1.03	0.0	-0.5	+2.4
4	0.0055	0.04	+0.5	+4.8	-12.4	+24.1

\* ATP P was determined by subtracting the direct P values from the P values obtained after 7 minutes hydrolysis in  $\text{N HCl}$  at  $100^\circ$ . No correction was made for partial hydrolysis of hexose diphosphate. Ester P represents the difficultly hydrolyzable P esters obtained by subtracting the P values after 7 minutes hydrolysis from the total acid-soluble P.

*Effect of Phosphocreatine on Glycolysis by Brain Homogenates*—Addition of phosphocreatine to brain homogenates stimulates lactic acid formation. This effect is partially due to the shift in pH toward the alkaline side, resulting from the breakdown of phosphocreatine, thus buffering the acids produced in the course of glycolysis. The large quantities of lactic acid produced quickly bring the pH below 6.0 and difficulties have been encountered in finding a suitable buffer which does not inhibit glycolysis when used in adequate concentrations. Finally a combination of 0.005 M ammonium phosphate buffer and 0.008 M potassium bicarbonate was found suitable. Under these conditions the addition of phosphocreatine in the absence of added  $\text{Na}^+$  has relatively little effect. However, in the presence

of  $\text{Na}^+$ , phosphocreatine was found effective in restoring the diminished glycolytic activity (Table V). This may be due to the maintenance of an effective ATP concentration by way of the reaction  $\text{ATP} + \text{creatine} \rightleftharpoons \text{ADP} + \text{phosphocreatine}$ , thus compensating for the rapidly acting ATPase in the brain tissue. No direct evidence is available for this view, which, however, is suggested by the marked increase of the P fraction after 7 minutes hydrolysis when phosphocreatine is present (Table V). According to this view the  $\text{Na}^+$  effect would be localized in the phosphorylation of the adenylic acid system, which is required for the phosphorylation of glucose

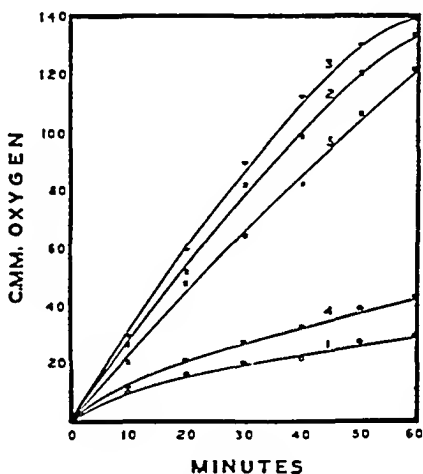


FIG. 1. Effect of sodium and phosphocreatine on oxygen uptake by brain homogenates. The brain preparations and solutions were as given in Table V. Oxygen uptake was measured in Warburg-Barcroft manometers. Curve 1, no glucose; Curve 2, 0.024 M glucose; Curve 3, 0.024 M glucose plus 0.0055 M phosphocreatine; Curve 4, 0.024 M glucose plus 0.08 M NaCl; Curve 5, 0.024 M glucose, 0.08 M NaCl plus 0.0055 M phosphocreatine.

and the hexose monophosphates. Boyer, Lardy, and Phillips (11) reported a stimulating effect of potassium on phosphorylation of the adenylic acid system in muscle preparations and also noted an inhibitory effect of  $\text{Na}^+$  in high concentrations.

On the other hand, Ohlmeyer and Ochoa (12), who first described an inhibitory influence of  $\text{Na}^+$  on the phosphate transfer from phosphopyruvic acid to glucose in yeast extracts, noted a much more pronounced inhibitory effect of  $\text{Na}^+$  in the presence of DPN, than with adenylic acid. Furthermore, in preliminary studies we have noted that  $\text{Na}^+$  is without effect not



only on the lactic acid production from hexose diphosphate but also from glucose-6-phosphate and fructose-6-phosphate. This would point to a localization of the  $\text{Na}^+$  inhibition on the phosphorylation of glucose rather than on the adenylic acid system, which is also required for the phosphorylation of hexose monophosphates.

The inhibitory effect of  $\text{Na}^+$  was also demonstrable under aerobic conditions. Fig. 1 represents a typical experiment. Oxygen uptake of the homogenates in the presence of glucose by far exceeded the optimal values for oxygen uptake by brain slices as recorded in the literature.  $\text{QO}_2$  values between 20 and 30 for the 1st hour were obtained.  $\text{Na}^+$  (0.08 M) strongly inhibited and phosphocreatine partially counteracted this inhibition (Fig. 1).

By the addition of DPN, ATP, nicotinic acid amide, and phosphocreatine and the exclusion of  $\text{Na}^+$ , homogenates were prepared from mouse brain which anaerobically regularly formed 7.5 mg. of lactic acid per 100 mg. of wet weight of brain per hour, and aerobically gave  $\text{QO}_2$  values between 20 and 30.

#### DISCUSSION

The presence of inhibitors to proteolytic enzymes in tissues has long been recognized. More recently the rôle of normally occurring inhibitors has been noted in the *in vitro* studies of carbohydrate and fatty acid metabolism (1, 13). The destruction of ATP and DPN by adenosinetriphosphatase (ATPase) and DPNase, which are widely distributed in animal tissues, interferes with many evaluations of enzyme concentrations and kinetic studies of impure enzyme preparations.

The presence of normal inhibitors complicates also the evaluation of the many conflicting reports on tissue glycolysis which have appeared in the literature. The use of NaCl, KCl, or water for the preparation of the enzyme solution may affect even the order of magnitude of the reaction rates. The choice of one or the other of these compounds can also determine the pace-maker in complex enzyme reactions such as those involved in glycolysis and thus simulate differences in the susceptibility to inhibitors. Studies on the specificity of inhibitors, such as glyceraldehyde, and claims of a pathway of glucose breakdown not involving phosphorylation by brain tissue must be reexamined in consideration of the presence of normal inhibitors.

Our own results (see Table V) make the presence of a non-phosphorylating pathway for glucose breakdown under our experimental conditions unlikely. Negligible amounts of glucose disappear in the presence of  $\text{Na}^+$  and with the addition of phosphocreatine the disappearance of inorganic P is proportional to the utilization of glucose.

Shortly before the present work was completed, Utter and Reiner (14)

and Klein (15) reported briefly on the rôle of nicotinic acid amide on brain metabolism. Utter and Reiner prepared actively glycolyzing rat brain homogenates by addition of hexose diphosphate, ATP, DPN, and nicotinic acid amide. These results have been fully confirmed by our studies on mouse brain. Hexose diphosphate, in common with phosphocreatine, counteracts the sodium inhibition. In our studies on the inhibition of the first steps of glycolysis it was necessary to avoid the use of hexose diphosphate because of its rôle as a substrate whose utilization is not inhibited by  $\text{Na}^+$ .

It may be pointed out that the well known effect of hexose diphosphate on the induction period of yeast fermentation may well be linked to a similar phenomenon and point to the presence of "normal" inhibitors in yeast.

#### SUMMARY

1. Two factors inhibiting glycolysis have been found in mouse brain homogenates. One of these is DPNase, the enzyme acting on DPN, which can be counteracted by addition of nicotinic acid amide, as demonstrated by Mann and Quastel. The second inhibitor was found to be  $\text{Na}^+$ , which can be counteracted by phosphocreatine.

2. The preparation of mouse brain homogenates, capable of producing from glucose anaerobically 7.5 mg. of lactic acid per 100 mg. of wet weight of brain per hour and having a  $\text{QO}_2$  of 20 to 30 is reported.

The authors wish to thank Dr. Severo Ochoa for his suggestions and help throughout this work.

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## THE INFLUENCE OF AGE AND DIET ON THE LIPID COMPOSITION OF THE RAT\*

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The variations in magnitude of the structural organization of protoplasm when referred to successive intervals of time (or as has been said, when equated with time) characterize the "phenomenon of growth" in living organisms (1). The chemical changes occurring in the body during growth, or with age, which is the reference point in time for the living organism, are of the utmost interest to the biochemist. A goal in this respect, as has been stated (2), is ultimately to "detect these changes and define them in terms of quantitative biochemistry."

The extensive studies reviewed by Moulton (3) and McCay (2) amply attest the marked alterations in the chemical composition of the body during growth and aging. Certain of the changes that occur with age may be the result of disease, and therefore difficult to evaluate. On the other hand, Moulton (3) concludes that, on the fat-free basis, "mammals show a rapid decrease in relative water content and increase in protein (nitrogen) and ash content from earliest life until the time of chemical maturity is reached." His calculations were made on a fat-free basis, since "the most striking change in the composition of mammals with increased age and development is the increase in the fat content" (3).

Recently, however, it has become increasingly apparent that not all fatty materials comprising animal substances are merely stores or depots of surplus energy, but that certain lipids are essential components of tissues and integral parts of the cellular structure (4, 5). Phospholipid, cholesterol, and cerebrosides are included in the latter category, neutral fat or triglyceride in the former. Thus, fundamental changes in the composition of animals coincident with growth and with aging may be obscured if the data are calculated on a fat-free basis, especially if "fat-free" implies, as originally, freedom from all lipid substances. For, as stated by Hammett (6), "Growth is not single, it is multiple, the combined expression of integrated developmental and incremental factors, processes and functions" and represents "the continued increase in living and essential structural substance." Part I of the present study deals with the effect

\* A preliminary report of Part I of this paper was presented before the Division of Biological Chemistry of the American Chemical Society at the 105th meeting at Detroit, Michigan, April 12-16, 1943.

of aging on the lipid composition of the body, with special emphasis on the differentiation of the total lipid into (a) the storage or reserve fat and (b) the "essential" or structural fatty components.

Growth is a function of two factors, heredity and environment (7), and, since the latter is amenable to variation, the effect of diet, one of the most significant environmental factors, also was studied. Part II deals with the effect of high and low fat diets on the lipid composition of the body during the most active period of growth. The influence of high and low fat diets on the composition of the reserve, or depot fat, has been studied extensively (8, 9). However, relatively little is known of the possible effect of such dietary variations on the relative distribution in the body of the "essential" lipid components.

### *Part I. Influence of Age on Lipid Composition of Body*

#### EXPERIMENTAL

From a colony of rats with the same heredity and dietary background (Rockland stock diet) new born rats and others which were 15, 45, and 70 days old were selected. The whole body of each animal (minus the contents of the intestines) was ground in a food chopper, frozen, and dried from the frozen state under a vacuum (10). The dried material was extracted with hot ethanol, then with ethyl ether. The final combined extract represented approximately a 3:1 mixture of alcohol-ether and was analyzed for choline, sphingomyelin, total and free cholesterol, galactose, and acetone-soluble glycerol.

For determination of total phospholipid, a portion of the alcohol-ether extract was evaporated at less than 50°, under reduced pressure, in a stream of nitrogen. The residue was extracted with petroleum ether, and the petroleum ether extract was analyzed for total phosphorus.<sup>1</sup> The analytical procedures have been published in detail elsewhere (11). From the analyses, total phospholipid, choline phospholipid, cephalin, lecithin, cholesterol esters, cerebroside, and neutral fat were calculated (5).

#### *Results*

The lipid composition of the body of new born rats and animals 15, 45, and 70 days old is presented in Table I. The total lipid content of the

<sup>1</sup> Total phosphorus of the alcohol-ether extract of the whole animal could not be used for the calculation of the total phospholipid, since, by repeated isolation of the phospholipid, it was shown that significant amounts of phosphorus other than phospholipid phosphorus were present in the alcohol-ether extract. This is true only for extracts made from the whole animal body and appears to result from the presence of bone. When bone was not present in the sample, we have found that the total phosphorus content of the alcohol-ether extract represents the phospholipid content.

new born rats was found to be 21.26 per cent, approximately one-fifth of the dry body weight. After 70 days, the total lipid had doubled in concentration and composed 40.72 per cent of the dry weight. However, this significant accumulation of fat during rapid growth does not represent the actual structural changes which are occurring in the tissues. The neutral fat, or storage lipid, fraction increased from 11.72 per cent at birth to 35.50 per cent of the dry weight at 70 days of age, a 3-fold increase. On the other hand, the essential lipid (total lipid minus neutral fat) which represents the structural lipid components showed a substantial drop in concentration from birth (9.54 per cent) to 70 days of age (5.22 per cent). Although all of the lipids composing the essential lipid follow the same trend, it is apparent that they do not decrease proportionally. Further-

TABLE I  
*Lipid Content of Rats at Different Ages*

The values are in per cent of dry weight

	New born, 28 animals	15 days old, 6 animals	45 days old, 4 animals	70 days old, 2 animals
Total lipid . . . .	21.26	26.68	38.23	40.72
Neutral fat . . . .	11.72	19.60	32.34	35.50
Essential lipid . . . .	9.54	7.08	5.89	5.22
Cerebrosides . . . .	1.15	1.17	1.07	1.04
Free cholesterol . . . .	0.95	0.49	0.34	0.25
Cholesterol esters . . . .	0.42	0.22	0.22	0.30
Phospholipid . . . .	7.02	5.20	4.26	3.63
Cephalin . . . .	1.37	2.13	2.50	1.82
Lecithin . . . .	5.22	2.60	1.42	1.35
Sphingomyelin . . . .	0.43	0.47	0.34	0.46

more, the composition of the phospholipid is markedly altered as growth proceeds. The proportion of lecithin in the total decreases, whereas cephalin increases.

To give a true picture of the structural changes occurring during growth, it is necessary to eliminate the variable influence of the storage fat. Therefore, in Table II, the essential lipid composition of the rat at different ages has been given on the basis of the neutral fat-free dry weight. The concentration of the essential lipid showed a downward trend between birth and 70 days, from 10.81 to 8.09 per cent. The most significant change occurred between birth and 15 days of age, with a drop from 10.81 to 8.80 per cent.

It is apparent, however, that the changes in the essential lipid components are not parallel. Both phospholipid and free cholesterol decreased in concentration with advancing age. Cerebrosides increased from a con-

centration of 1.30 per cent at birth to 1.61 per cent at 70 days of age. The cholesterol ester content at birth was 0.48 per cent of the neutral fat-free dry weight; after 15 days it had dropped to 0.27 per cent, but at 70 days it had risen to 0.47 per cent.

The partition of the phospholipid shows the marked changes that occur in this lipid fraction as growth progresses. Lecithin and cephalin showed inverse trends up to 45 days of age; the lecithin decreased and the cephalin

TABLE II

*Essential Lipid Composition of Rats at Different Ages*

The values are in per cent of neutral fat-free dry weight.

	New born	15 days old	45 days old	70 days old
Essential lipid.....	10.81	8.80	8.70	8.09
Cerebrosides.....	1.30	1.46	1.58	1.61
Free cholesterol.....	1.08	0.61	0.50	0.39
Cholesterol esters .....	0.48	0.27	0.33	0.47
Phospholipid.....	7.95	6.46	6.29	5.62
Cephalin.....	1.55	2.65	3.69	2.82
Lecithin.....	5.91	3.23	2.10	2.09
Sphingomyelin.....	0.49	0.58	0.50	0.71

TABLE III

*Percentage of Lipid Distribution*

		New born	15 days old	45 days old	70 days old
Total lipid	Neutral fat	55	73	85	87
	Essential lipid	45	27	15	13
Essential lipid	Cerebrosides	12	17	18	20
	Free cholesterol	10	7	6	5
	Cholesterol esters	4	3	4	6
	Phospholipid	74	73	72	69
Total phospholipid	Cephalin	20	41	59	50
	Lecithin	74	50	33	37
	Sphingomyelin	6	9	8	13

increased in concentration. After 70 days lecithin remained unchanged, whereas the cephalin showed a significant drop in concentration. Although sphingomyelin showed no consistent trend, the concentration changed from 0.49 per cent at birth to 0.71 per cent at 70 days of age.

The effect of growth on the lipid pattern is presented in Table III. The tremendous changes in the lipid composition of the body are striking. The neutral fat fraction composed 55 per cent of the total lipid at birth, whereas

at 70 days it made up 87 per cent of the total. The largest component of the essential lipid, phospholipid, comprised 74 per cent of the total at birth and fell to 69 per cent at 70 days of age. In contrast, cerebrosides, the second largest constituent of the essential lipid, were 12 per cent of the total at birth, but rose to 20 per cent at 70 days. Although the cholesterol fractions are minor components of the essential lipid, at least quantitatively, they show significant alterations during growth. In percentage of essential lipid, free cholesterol decreased from 10 per cent at birth to 5 per cent of the total at 70 days, while the cholesterol esters were 4 per cent at birth, but composed 6 per cent of the total at 70 days of age.

Similar to that of the essential lipid, the pattern of the phospholipid components shows marked changes with advancing age. Cephalin made up one-fifth of the total at birth but increased to one-half at 70 days of age. Likewise, the smallest fraction of the phospholipid, sphingomyelin, increased from 6 to 13 per cent. Consequently, the lecithin fraction which comprised three-quarters of the total phospholipid at birth made up little more than one-third (37 per cent) of the total at 70 days of age.

## *Part II. Influence of Diet on Lipid Composition of Body<sup>2</sup>*

### EXPERIMENTAL

At weaning (22 days of age) four male rats were taken from each of fifteen litters and litter mates were placed in different groups, making four groups of fifteen rats each. The animals were distributed so that the total weights of each of the groups were approximately equal. The animals in one group, designated *control*, were killed immediately, the contents of the intestines removed, the whole body ground in a food chopper, frozen, and dried from the frozen state under a vacuum (10). Each of the remaining three groups was placed on a special diet, and each rat was fed 3000 calories, after which it was killed and the whole body ground and dried as described. The three diets contained the same ingredients casein, sucrose, salt mixture,<sup>3</sup> Crisco, dextrin, and vitamin supplements. The only variations were in the amounts of Crisco and dextrin, which together furnished 77.2 per cent of the caloric intake and were altered isodynamically to produce high or low fat and carbohydrate diets. In the group designated as *adequate*, Crisco and dextrin each furnished 38.6 per cent of the total caloric intake; in the group designated *high fat*, Crisco

<sup>2</sup> This is the initial report of a series of studies upon the effects of diet upon growth and the approximate composition of animal tissues.

<sup>3</sup> Wesson's salt mixture (12).

<sup>4</sup> Dietary supplements (three times weekly): ryzamin-B No. 2, 200 mg.; Lilly's liver-stomach extract, 100 ml.; White's concentrated cod liver oil, 50 mg.; wheat germ oil, 40 ml.; linoleic acid, 20 mg.; and choline chloride, 35 mg.



and dextrin furnished, respectively, 67.9 and 9.3 per cent of the calories; and in the group designated *high carbohydrate*, these foods furnished 9.3 and 67.9 per cent, respectively. The amounts of all the remaining dietary ingredients were the same in each diet. Feeding was controlled (the food intake being weighed three times per week) so that all animals consumed their diets in approximately the same number of days (average, 66). Each animal was kept alone in a false bottom cage and allowed water *ad libitum*. The vitamin supplements were fed to each animal, separately, three times weekly. After the animals were killed at the end of the dietary period, the whole bodies were ground in a food chopper, frozen, and dried. 10 per cent of the dry material from each rat was used to make four composites, each composite representing one of the diet groups. The composites were extracted and the lipids determined, as described in Part I.

TABLE IV

*Effect of Diet on Lipid Composition of Rats*

The values are in per cent of dry weight.

	Control	Adequate diet	High fat diet	High carbohydrate diet
Total lipid.....	16.58	44.80	49.10	36.01
Neutral fat.....	8.71	40.56	45.16	32.28
Essential lipid.....	7.87	4.24	3.94	3.73
Cerebrosides.....	1.23	1.59	1.40	0.81
Free cholesterol.....	0.43	0.15	0.14	0.15
Cholesterol esters.....	0.75	0.14	0.14	0.25
Phospholipid.....	5.46	2.36	2.26	2.52
Cephalin.....	2.49	0.84	0.97	1.01
Lecithin.....	2.30	1.21	0.93	1.13
Sphingomyelin.....	0.67	0.31	0.36	0.38

*Results*

The lipid distributions of the composites of whole bodies of rats before (control) and after ingestion of 3000 calories of the experimental diets (adequate, high fat, and high carbohydrate) are presented in Table IV. The results show that all of the experimental diets produced a large storage of fat, although it is evident that much less fat was stored on the high carbohydrate than on the other diets. The high carbohydrate diet appears to have influenced the lipid composition of the body with respect to such essential lipid components as cerebrosides and cholesterol. However, the differences in neutral fat are such as to obscure the significance of these changes. Therefore, the distribution of the essential lipid is given in Table V in terms of neutral fat-free dry weight.

No significant differences in the structural (essential) lipid distribution

in the bodies of rats fed the adequate or high fat diets are evident. The data show a decreased concentration of the phospholipid and cholesterol components and an increased content of cerebrosides over that of the control. These changes are typical of advancing age, as shown in Part I, and are not the result of diet. However, the high carbohydrate diet appears to have produced marked differences in the essential lipid distribution.

The phospholipid component appears to be least affected by the high carbohydrate diet, although the concentration (3.72 per cent) was lower than that for the adequate (3.97 per cent) or the high fat diet (4.12 per cent). The decreased concentration that occurs as growth proceeds is apparent, inasmuch as the controls contained 5.98 per cent phospholipid. The most significant effect appears to have been on the content of cere-

TABLE V

*Effect of Diet on Essential Lipid Composition of Rats*

The values are in per cent of neutral fat-free dry weight.

	Control	Adequate diet	High fat diet	High carbohydrate diet
Essential lipid.....	8.62	7.13	7.19	5.51
Cerebrosides.....	1.35	2.67	2.55	1.20
Free cholesterol.....	0.47	0.25	0.26	0.22
Cholesterol esters.....	0.82	0.24	0.26	0.37
Phospholipid.....	5.98	3.97	4.12	3.72
Cephalin.....	2.73	1.41	1.77	1.49
Lecithin.....	2.52	2.04	1.69	1.67
Sphingomyelin.....	0.73	0.52	0.66	0.56

brosides. The animals contained only 1.20 per cent cerebrosides in contrast to 1.35 per cent for the controls and 2.67 and 2.55 per cent for the adequate and high fat groups, respectively. Likewise, the concentration of free cholesterol was less in the high carbohydrate-fed rats than in the other dietary groups. Contrariwise, cholesterol ester synthesis appears to have been greater on the high carbohydrate diet.

The lipid distribution pattern is given in Table VI. It is apparent that the type of diet had little or no effect on the proportion of essential lipid and storage lipid or fat. In all diet groups 90 per cent or more of the total lipid was neutral fat, despite the fact that there was much less total lipid, and therefore neutral fat (Table IV) in the animals fed the high carbohydrate diet than in those fed either the adequate or high fat diets. The patterns of the components comprising the essential lipid, however, demonstrate the marked differences between the high carbo-

hydrate diet and the other dietary régimes. Those for both the adequate and the high fat diets are practically identical. A little more than one-half (56 to 57 per cent) of the essential lipid material was phospholipid and a little more than one-third (36 to 37 per cent) was cerebroside. The remaining 7 per cent was about equally divided between free cholesterol (4 per cent) and cholesterol esters (3 per cent). In contrast, the essential lipid pattern of the animals fed the high carbohydrate diet showed 67 per cent phospholipid and only 22 per cent cerebroside. Further, although the free cholesterol value was similar (4 per cent), the cholesterol ester content was more than double (7 per cent) that for any of the other dietary groups. In fact, the essential lipid pattern of the high carbohydrate group is little different from that of the control group.

TABLE VI  
*Percentage of Lipid Distribution*

		Control	Adequate diet	High fat diet	High carbohydrate diet
Total lipid	Neutral fat	53	91	92	90
	Essential lipid	47	9	8	10
Essential lipid	Cerebrosides	16	37	35	22
	Free cholesterol	5	4	4	4
	Cholesterol esters	10	3	4	7
	Phospholipids	69	56	57	67
Total phospholipid	Cephalin	46	36	43	40
	Lecithin	42	51	41	45
	Sphingomyelin	12	13	16	15

The distribution of the individual phospholipids in terms of total phospholipid shows little significant alteration as the result of diet differences. Sphingomyelin composed 12 to 16 per cent of the total for all groups and the remainder was about equally distributed between cephalin and lecithin.

#### DISCUSSION

The effect of aging and diet on the lipid composition of the rat has been studied during the period of most rapid growth by that animal species. The experiments in Part I encompassed the period from birth to 70 days of age; those of Part II covered the period from 22 to approximately 88 days of age, since the average time the animals took to consume 3000 calories was 66 days.

The results in Part I show the tremendous increase in fat content of the body during growth and aging, and confirm previous studies on rats

and other species. The changes in body composition are marked by a 2-fold increment in the proportion of total lipid to total dry weight, from one-fifth of the dry weight at birth to two-fifths at 70 days. That this seemingly physiological alteration in body composition is not greatly limited by requiring the body to secure the fatty substances from energy sources other than fat (in this case largely by synthesis from carbohydrate) is demonstrated by the results of feeding a high carbohydrate diet.

However, the present experiments were not designed to investigate this particular phase of fat metabolism, which has been extensively studied by others. Rather, the purpose was to determine the influence of aging and diet upon the lipid components essential to cell structure and function. To accomplish this, it is necessary to separate the storage lipid and the essential lipid fractions in the total lipid. The data amply demonstrate the importance of such a differentiation. The changes which occur in the total lipid content of the body in response to either aging or diet are a reflection of the alterations in the neutral fat fraction, since this component usually composes, under a wide variety of conditions, 50 to 90 per cent of the total lipid. Therefore, for the study of the lipid structural changes in the tissues, it is necessary to base the calculations on the neutral fat-free composition.

On that basis, certain fundamental changes in the essential lipid composition of the body take place during growth and development. The total phospholipid and free cholesterol concentrations decrease, whereas that of cerebroside increases. Cholesterol esters are highest at birth, lowest at 15 days of age, and by 70 days return to approximately the concentration at birth. For phospholipid and cholesterol the results indicate that although these components of the essential lipid are being synthesized, since in absolute amounts the body of a 70 day-old rat contains more phospholipid and cholesterol than that of the new born rat, the rate of synthesis is less than that of other structural elements such as protein. On the other hand, the synthesis of cerebroside occurs at a higher rate than for other structural elements. The same is true for the cephalin portion up to 45 days of age. For cholesterol esters, formation is less rapid than for the whole neutral fat-free body substance, for the first 15 days of age, but after this time accumulation of this lipid fraction is at a higher rate.

These changes in the lipid composition of the body present a composite picture of the alterations occurring in the individual organs and tissues composing the body, as well as reflect the changes in the relative proportions of the various types of body tissues as growth progresses. The importance of the concentration of essential cellular lipids in various types of body tissues already has been indicated (5). Although the brain and other neural tissues rank first in lipid concentration, of the various

tissue systems of the body, muscle tissues are among those which contain the lowest amounts. Thus the musculature might be expected to exert a preponderant influence upon the lipid composition because of the large amounts present in the body.

The musculature of the rat is approximately 24 per cent of the body weight at birth, and 41 per cent at 70 days of age (13); therefore, the marked changes in the concentration of certain of the essential lipid components during growth may be a reflection of growth and expansion of the muscular system. Inasmuch as the phospholipid and free cholesterol concentrations in muscle (with the exception of heart muscle) are relatively low (5), increase in this type of tissue would consequently lower the concentration of these lipid fractions in the whole. Also, muscles contain a relatively high content of cerebrosides (5), and therefore the increasing concentration of cerebrosides in the body with advancing age may be, likewise, a reflection of the proportional increase in musculature. The increase in cephalin concentration can be explained similarly. An analogous explanation for the changes observed in the cholesterol ester fraction would not seem to be justified, due to the very small amounts of this lipid component in muscle tissue. Rather, since notable concentrations of cholesterol esters are normally found in the body only in the adrenals, liver, and blood plasma, the growth of these tissues may be responsible for the changes occurring in the cholesterol ester concentration of the body during growth. As an example of this possibility, the blood volume of a new born rat has been given as about 0.25 ml. (13); after 15 days it has increased to about 0.54 ml., by 45 days it is little over 4 ml., and at 70 days nearly 8 ml.

The results in Part II show that diets in which the main sources of calories are equally divided between fat and carbohydrate, or those in which fat furnished most of the calories, had little influence on the structural lipid content or pattern. The alterations which occurred, such as a decreased concentration of phospholipid and an increased content of cerebrosides, are typical of the changes which take place in the essential lipid content of the body during growth. However, feeding a diet in which the major portion of the calories was derived from carbohydrate yielded different results. Synthesis of cerebrosides and free cholesterol appears to be impaired on this diet, and that of cholesterol esters enhanced. Some explanation for these results may be evident from the work of Treadwell and Eckstein (14) who have concluded that "the fat content of the diet is intimately associated with the synthesis of cholesterol in the young white rat." Larger amounts of cholesterol were synthesized on a high than on a low fat diet.

These studies of the whole animal body indicate the importance of

separating the lipids of the body into separate categories: (a) storage lipid or neutral fat and (b) essential, or structural, lipids, in determining the effect of growth or diet on body composition. Alterations in the essential lipid pattern of the tissues and cells of the body are not shown by changes in total lipid composition, which reflect the variations in the relatively large quantity of neutral fat. Neutral fat, although important from the standpoint of energy storage and reserve, is unessential in the cell structure.

As the body ages and growth proceeds, the pattern of essential lipid composition is altered significantly, probably as the result of the alterations in the essential lipid composition of individual tissues and the changes in the relative proportions of the various tissues present in the body. Moreover, this essential lipid pattern may be influenced by diet, for restriction of the proportion of calories derived from fat may retard the synthesis of cerebroside and alter the proportions of free and combined cholesterol.

#### SUMMARY

The lipid distribution (phospholipid (cephalin, lecithin, and sphingomyelin), free and combined cholesterol, cerebroside, and neutral fat) was determined in the whole bodies of new born rats and animals 15, 45, and 70 days old, and in the whole bodies of control rats (22 days of age) and litter mates after each had ingested 3000 calories of an adequate, high fat, or high carbohydrate diet.

The importance of separating the lipids of the body into (a) neutral, or storage lipid, fat and (b) essential, or structural, lipid has been demonstrated. Alterations in the essential lipid composition of the body during growth are not reflected in the changes occurring in total lipid composition, for the latter reflects the variations in the neutral fat which composes the largest portion of the total (50 to 90 per cent).

The essential lipid pattern is altered significantly during growth and development. The concentrations of phospholipid and free cholesterol decrease, whereas those of cerebroside and cholesterol esters increase, probably not only as the result of changes in the composition of individual tissues but also because of the changes in the relative proportions of the various tissues present in the body.

Although diets in which 40 to 70 per cent of the calories were derived from fat had no influence on the essential lipid composition of the body, a diet in which less than 10 per cent of the calories was furnished by fat produced an apparently significant alteration. On the latter diet the rats contained less cerebroside and free cholesterol and more esterified cholesterol.

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# THE EFFECT OF GROWTH ON THE LIPID COMPOSITION OF RAT TISSUES\*

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Growth has been characterized as "the continued increase in living and essential structural substance" (1). The rapid accumulations of nitrogen (protein) and ash (minerals) are distinctive features of the chemical changes occurring in the mammalian body coincident with the phenomenon of growth (2). Minerals are preeminent structural components of the skeleton of the body. Similar prominence has been attributed to protein in the construction of the "soft" tissues. Equally important as essential structural constituents, however, are the lipids (3).

Normally, the major portion of the lipid in the body is triglyceride or neutral fat which serves as an energy depot or reserve, the concentration depending upon general "nutriture." However, the concentration of that portion of the body lipid which is essential in the cell structure (phospholipid, cholesterol, and cerebrosides) is in large measure independent of the diet and dependent upon the age, or size, of the organism (4). Both the concentration and the distribution pattern of the essential lipids vary among the different tissues of the body in proportion to the extent and variety of the physiological activities (3, 5) of the tissues.

Although an increase in the total fat content of the animal body is a characteristic change with increased age, this does not represent the changes in essential lipid composition which occur as the result of growth and development. Rather, the increased total lipid reflects an augmented reserve deposit of neutral fat. As has been indicated (4), alterations in the essential lipid composition may reflect fundamental structural changes in the tissues, as well as changes in the relative amounts of the various tissues during growth.

The present study was designed to investigate the lipid composition of various organs and tissues relative to the structural changes associated with growth.

## EXPERIMENTAL

Male rats, 15, 45, and 70 days of age, were selected from the same stock and with similar dietary backgrounds (Rockland rat diet). The body of

\* A preliminary report of this study was presented before the Division of Biological Chemistry of the American Chemical Society at the 105th meeting at Detroit, Michigan, April 12-16, 1943.



each animal was opened under ether anesthesia, and after the section of the aorta, as much blood as the heart would pump out was sponged away. The heart, thymus, lungs, liver, kidneys, spleen, testes, brain, and muscle tissue from the legs and back were dissected and placed in separate bottles. The tissues, after desiccation from the frozen state under a vacuum (6), were extracted with hot ethanol, then with ethyl ether. The final combined extract represented approximately a 3:1 mixture of alcohol-ether and was analyzed for total phosphorus, choline, sphingomyelin, total and free cholesterol, galactose, and acetone-soluble glycerol. Total phospholipid, choline phospholipid, cephalin, lecithin, cholesterol esters, cerebrosides, and neutral fat were calculated from the analytical results (3). Details of the analytical procedures have been published (7).

### *Results*

The lipid composition of the tissues from animals 15, 45, and 70 days old is presented in Table I. The data for the different tissues are arranged in the order of descending concentration of essential lipid at 15 days of age. All of the tissues except thymus and skeletal muscle show a consistent increase in essential lipid content during growth from 15 to 70 days of age. These changes are a reflection of those of the major component of the essential lipid, the phospholipid, since in every instance they are parallel. However, the other components of the essential lipid, cerebrosides and cholesterol, change independently, not only with respect to each other, but also to the total essential lipid as well, during the same period of growth.

Cerebrosides increase more than 2-fold in the brain, testes, and skeletal muscle, whereas in the heart cerebrosides drop from 2.32 per cent at 15 days to 1.37 per cent at 70 days of age. Free cholesterol shows a definite increase with age in the brain and lung and a decrease in the heart and thymus. For cholesterol esters during growth, there was a fall in concentration in the brain, heart, and skeletal muscle, and a rise in the testes, thymus, and spleen.

For the tissues studied, except skeletal muscle, of animals 15 and 45 days old, the neutral fat fraction is a minor part of the total lipid in the tissue and as growth proceeds, the neutral fat decreases.

The changes in the phospholipid distribution in the tissues during growth are given in Table II. In every tissue except lung and skeletal muscle the content of the cephalin fraction parallels that of the total phospholipid. For the total of the choline phospholipids, the kidney, lung, and testes show a higher content at 70 days of age than at 15, but for the other tissues the opposite is true. The lecithin component appears to follow a parallel course, although the concentration of sphingomyelin seems to vary

independently of either the choline phospholipids or the total phospholipid. The brain, kidney, lung, and spleen show a significant increase in sphingomyelin concentration during growth from 15 to 70 days of age, while the

TABLE I  
*Lipid Composition of Rat Tissues*

The values are in per cent of dry weight

Tissue	Age	No of animals	Phospholipid	Cerebrosides	Free cholesterol	Cholesterol esters	Essential lipid	Neutral fat	Total lipid
	<i>days</i>								
Brain	15	107	21.34	3.77	4.44	0.27	29.82	2.80	32.62
	45	13	26.43	8.52	6.57	0.14	41.66	2.14	43.80
	70	6	27.19	8.42	7.05	0.02	42.68	1.95	44.63
Heart	15	88	12.85	2.32	0.77	0.46	16.40	2.90	19.30
	45	13	14.16	2.14	0.43	0.28	17.01		
	70	6	15.38	1.37	0.45	0.21	17.41		
Kidney	15	107	11.99	1.20	1.16	0.84	15.19	4.43*	19.62
	45	13	14.15	1.58	0.88	1.15	17.76	3.57	21.33
	70	6	15.19	1.30	1.00	0.94	18.43	3.16	21.59
Lung	15	107	10.75	0.86	1.00	1.44	14.05	6.92	20.97
	45	13	12.49	1.16	1.15	0.99	15.79	5.51	21.30
	70	6	13.75	0.91	1.43	1.02	17.11	4.75	21.86
Testes	15	88	10.32	1.86	1.31†	0.24†	13.73	5.96	19.69
	45	13	14.54	3.67	0.82	0.63	19.66	2.97	22.63
	70	6	14.97	3.96	0.82	0.66	20.41	2.77	23.18
Liver	15	107	11.67	0.12	0.27	1.44	13.50	6.35	19.85
	45	13	12.64	0.62	0.31	0.57	14.14	3.29	17.43
	70	6	13.90	0.13	0.29	0.66	14.98	5.22	20.20
Thymus	15	88	9.72	1.34†	0.57†	0.46†	12.09	6.89	18.98
	45	13	11.66	0.52	0.28	0.62	13.08		
	70	6	10.75	1.14	0.24	0.61	12.74		
Spleen	15	88	6.93	0.60	1.34	0.08†	8.95	2.74	11.69
	45	13	9.57	0.28	0.93	0.52	11.30	2.11	13.41
	70	6	10.76	0.84	1.08	0.51	13.19		
Skeletal muscle	15	116	5.95	1.45	0.20‡	0.61‡	8.21	19.58	27.79
	45	13	5.17	2.41	0.09	0.17	7.84	8.77	16.61
	70	6	8.57	3.57	0.12	0.14	12.40	3.44	15.84

\* 88 of the 107 rats used

† 19 different rats

‡ 88 of the 116 rats

muscle tissues, both heart and skeletal, show a decrease. In contrast, the sphingomyelin content in liver, testes, and thymus appears to be relatively unchanged during this period of growth.

The lipid distribution of the various tissues is presented in Table III.

It is apparent that the major portion of the total lipid is essential lipid. With the exception of muscle, in which only 30 per cent of the total lipid was essential lipid at 15 days of age, over 60 per cent of the total at 15 days and over 70 per cent at 70 days of age were essential lipid. Although the data are incomplete, for those tissues in which the neutral fat could

TABLE II  
*Phospholipid Distribution in Rat Tissues*

The values are in per cent of dry weight.

Tissues	Age	Total phospho-lipid	Cephalin	Choline phospholipids		
				Total	Lecithin	Sphingo-mycin
	<i>days</i>					
Brain.....	15	21.34	10.37	10.97	7.04	3.93
	45	26.43	17.07	9.36	5.37	3.99
	70	27.19	18.05	9.14	4.87	4.27
Heart.....	15	12.85	5.53	7.32	6.30	1.02
	45	14.16	6.15	8.01	7.59	0.42
	70	15.38	9.01	6.37	5.89	0.48
Kidney.....	15	11.99	5.83	6.16	5.21	0.95
	45	14.15	7.19	6.96	5.19	1.77
	70	15.19	7.40	7.79	5.96	1.83
Lung.....	15	10.75	3.73	7.02	5.04	1.98
	45	12.49	3.22	9.27	7.17	2.10
	70	13.75	4.63	9.12	6.53	2.59
Testes.....	15	10.32	3.19	7.13	6.03	1.10
	45	14.54	7.39	7.16	6.09	1.06
	70	14.97	7.74	7.23	6.19	1.04
Liver.....	15	11.67	5.18	6.49	6.05	0.44
	45	12.64	6.61	6.03	5.55	0.48
	70	13.90	7.70	6.20	5.85	0.35
Thymus.....	15	9.72	5.23	4.49	3.63	0.86
	45	11.66	7.27	4.39	3.73	0.66
	70	10.75	6.75	4.00	3.28	0.72
Spleen.....	15	6.93	1.38	5.55	5.03	0.52
	45	9.57	5.20	4.37	3.44	0.93
	70	10.76	6.17	4.59	3.48	1.11
Skeletal muscle.....	15	5.95	1.42	4.53	4.05	0.48
	45	5.17	2.71	2.46	2.30	0.16
	70	8.57	4.84	3.73	3.56	0.17

be determined there was, except in liver, a consistent drop in the percentage of neutral fat and a corresponding increase in essential lipid as the tissues grew.

In animals 15 days old, over 70 per cent of the essential lipid is composed of phospholipid. In brain, testes, and muscle, the proportion of phos-

pholipid in essential lipid is lower at 70 days than at 15 days of age, but in the remaining tissues investigated the per cent of phospholipid is higher at the 70 day age level.

TABLE III  
*Distribution of Lipids in Rat Tissues*

Tissue	Age	Per cent of total lipid		Per cent of essential lipid				Per cent of total phospholipid			
		Neutral fat	Essential lipid	Cerebrosides	Free cholesterol	Cholesterol esters	Phospholipid	Cephalin	Choline phospholipid		
									Total	Lecithin	Sphingomyelin
	days										
Brain.....	15	8.6	91.4	12.6	14.9	0.9	71.6	48.6	51.4	33.0	18.4
	45	4.9	95.1	20.5	15.8	0.3	63.4	64.6	35.4	20.3	15.1
	70	4.4	95.6	19.7	16.5	0.1	63.7	66.4	33.6	17.9	15.7
Heart.....	15	15.0	85.0	14.1	4.7	2.8	78.4	43.1	56.9	49.0	7.9
	45			12.6	2.5	1.7	83.2	43.4	56.6	53.6	3.0
	70			7.9	2.6	1.2	88.3	58.6	41.4	38.3	3.1
Kidney.....	15	22.6	77.4	7.9	7.6	5.5	79.0	48.6	51.4	43.5	7.9
	45	16.7	83.3	8.9	4.9	6.5	79.7	50.8	49.2	36.7	12.5
	70	14.6	85.4	7.1	5.4	5.1	82.4	48.7	51.3	39.2	12.1
Lung.....	15	33.0	67.0	6.1	7.1	10.3	76.5	34.7	65.3	46.9	18.4
	45	25.9	74.1	7.3	7.3	6.3	79.1	25.8	74.2	57.4	16.8
	70	21.7	78.3	5.3	8.3	6.0	80.4	33.7	66.3	47.5	18.8
Testes.....	15	30.3	69.7	13.5	9.5	1.8	75.2	30.9	69.1	58.4	10.7
	45	13.1	86.9	18.7	4.2	3.2	73.9	50.8	49.2	41.9	7.3
	70	11.9	88.1	19.4	4.0	3.2	73.4	51.7	48.3	41.4	6.9
Liver.....	15	32.0	68.0	0.9	2.0	10.7	86.4	44.4	55.6	51.8	3.8
	45	18.9	81.1	4.4	2.2	4.0	89.4	52.3	47.7	43.9	3.8
	70	25.8	74.2	0.9	1.9	4.4	92.8	55.4	44.6	42.1	2.5
Thymus.....	15	36.3	63.7	11.1	4.7	3.8	80.4	53.8	46.2	37.3	8.9
	45			4.0	2.1	4.7	89.2	62.3	37.7	32.0	5.7
	70			8.9	1.9	4.8	84.4	62.8	37.2	30.5	6.7
Spleen.....	15	23.4	76.6	6.7	15.0	0.9	77.4	19.9	80.1	72.6	7.5
	45	15.7	84.3	2.5	8.2	4.6	84.7	54.3	45.7	36.0	9.7
	70			6.4	8.2	3.9	81.5	57.4	42.6	32.3	10.3
Skeletal muscle.....	15	70.5	29.5	17.7	2.4	7.4	72.5	23.8	76.2	68.1	8.1
	45	52.8	47.2	30.7	1.2	2.2	65.9	52.4	47.6	44.5	3.1
	70	21.7	78.3	28.8	1.0	1.1	69.1	56.5	43.5	41.5	2.0

In all the tissues except lung and spleen, cerebrosides constitute the second largest fraction of the essential lipid. In these two organs the cholesterol fraction comprises a larger percentage of the total. The cere-

broside content of the brain, testes, and skeletal muscle shows a marked proportional increase from 15 to 70 days of age, but for the heart there is a significant decrease. The brain and lung are the only tissues in which free cholesterol shows a proportional increase with the advance in age. In most tissues there is a drop in the percentage of cholesterol esters in the essential lipid during growth, but in the testes, thymus, and spleen there is an increase.

The percentage distribution of the phospholipids, which is given in Table III, shows that, during growth, in all tissues except kidney and lung there is a consistent increase in the proportion of cephalin to total phospholipid and a corresponding decrease in total choline phospholipid. The trend of the choline phospholipid is largely a reflection of the changes in the amounts of lecithin, in every instance except for the heart. Sphingomyelin shows a proportional decrease in muscle (both heart and skeletal) and in the testes, whereas in kidney and spleen there appear to be significant increases.

#### DISCUSSION

The importance of separating the tissue lipids into separate categories, essential lipid and storage lipid, has been indicated (3). Likewise, the significance of this distinction relative to the lipid changes in the body as a whole during growth was pointed out (4). Similarly, although in the present investigation the lipid composition of the various organs has not been presented on the neutral fat-free basis, since this fraction is a minor component of the total lipid in the tissues studied and does not obscure the fundamental changes in the lipids essential in their structure, yet the importance of differentiating between an essential fraction and a depot, or reserve, fraction among the components of the total is evident. Skeletal muscle furnishes the best example, although liver is equally illustrative. For the muscle, the total lipid changes from a high of 27.79 per cent at 15 days to a low of 15.84 per cent at 70 days of age. This is a reflection of the marked changes in the triglyceride or neutral fat component, which drops from 19.58 per cent at 15 days of age to 3.44 per cent at 70 days. The concentration of the essential lipid, however, falls slightly from 15 to 45 days of age (8.21 to 7.84 per cent) and markedly increases by 70 days to 12.40 per cent.

From the standpoint of lipid composition, the growth of the tissues studied is characterized by an increase in the concentration of the essential lipid (Table I). Thymus appears to be a special case. This particular organ, although exhibiting a slightly higher content of essential lipid at 70 days of age (12.74 per cent of the dry weight) than at 15 days (12.09 per cent), actually contains a greater concentration at 45 days (13.08 per

cent). In this respect, it is well known that the thymus of the rat retrogresses after puberty, and as shown by Donaldson (8), the thymus is the only organ among the tissues here considered which does not show an absolute weight increase throughout life.

The increase in essential lipid content of the tissues, concomitant with growth, is a reflection of the change in the phospholipid component which comprises 65 to 90 per cent of the total (Table III). Furthermore, except for lung tissue from animals 45 days old, the phospholipid reflects the increase in cephalin (Table II). Quantitatively, therefore, the fundamental change in lipid composition during growth, common to all the tissues studied, appears to be an increase in cephalin concentration.

In contrast, the other essential lipid components exhibit changes which vary among the different organs and which are probably related to specific functional and structural changes in a particular tissue during a phase of growth and development. For example, the cerebroside content of the brain increases more than 2-fold between 15 and 45 days of age, and increases in testes and skeletal muscle up to 70 days, whereas the cerebroside content of heart muscle decreases nearly one-half, between 15 and 70 days of age. The only organ which shows a rise in free cholesterol content consistent with growth, exclusive of the brain, is the lung; both heart and skeletal muscles show a marked drop.

It may be significant that the changes noted for the free cholesterol in brain, lung, and heart and skeletal muscle are paralleled by similar changes in the sphingomyelin components of the phospholipids. This parallelism does not follow for all tissues, however, since kidney and spleen register consistent increases in sphingomyelin content during growth, whereas for free cholesterol the changes, although minor, tend towards a lower concentration.

From the lipid pattern of the various organs (Table III) it is possible to note the significance of certain essential lipid constituents relative to a particular organ, which might not be apparent from the figures on concentration. Brain has the lowest relative percentage of phospholipid and the highest of free cholesterol, in the essential lipid. Skeletal muscle contains the highest percentage content of cerebrosides in essential lipid, followed by brain and testes, whereas liver has the lowest. The growth of the tissues accentuates these percentage patterns.

There is a general tendency in the phospholipid pattern for the relative percentage of cephalin to increase and that of lecithin to decrease. As was noted (3), the relative percentage of sphingomyelin is highest in the lung and lowest in the muscles.

With respect to the individual tissues, several points appear to merit emphasis. The brain, being the master neural tissue, contains the highest

concentration of the essential lipid components. These, except for lecithin and cholesterol esters, increase in concentration during the growth period (15 to 70 days) covered in this study. Among the early investigations of the chemical differentiation of the brain during growth, those of Koch (9) are of classic interest. Relatively, however, the brain contains the lowest percentage phospholipid (of essential lipid) of any tissue. Furthermore, the relative percentage of sphingomyelin is higher in lung than in brain. Also, cerebrosides are greater in skeletal muscle and the relative amount in testes is equal to that in brain. Therefore, from the viewpoint of function, it would appear that, although cephalin is of relatively great importance to the brain, phospholipid as a whole is not, in comparison to cerebrosides and free cholesterol and to the phospholipid in other organs. Likewise, cerebrosides appear to be much more significant in skeletal muscle, relatively, than in brain. However, free cholesterol appears to be relatively unimportant in muscle, particularly in skeletal muscle. Similarly, sphingomyelin is relatively unimportant in muscle tissue, but appears to be quite significant in the lung, brain, and kidney.

Judged from the changes in both the quantity of the essential lipid components and the lipid pattern of the various tissues during growth, the fraction of the phospholipid designated as cephalin is the common structural lipid component of all the tissues investigated. The fact that neural tissue contains such large quantities of the essential lipids which exhibit a characteristic expansion during growth and development supports the view (10, 11) that at least part of the lipids of a tissue is related to the neural structures in that tissue. That not all the essential lipid is so related is indicated by the characteristic developmental patterns among the various tissues. In no case are there any which, either quantitatively or qualitatively, are exactly similar to that of neural tissue (brain).

For example, such characteristic lipid changes as are exemplified by the cerebrosides of skeletal muscle in contrast to that of cardiac muscle imply that the essential lipid components are not merely the reflection of a neural mechanism in the tissue, but are structural components of the tissue cells associated with growth and a specific functional development. Characteristic physiological properties of skeletal muscle (12) are "ability to contract quickly and strongly and to be tetanized, but not spontaneously active; capable of large expenditures of energy; single nerve supply, which is from the outside." For cardiac muscle, they are "spontaneous rhythmic activity; absolute refractory period, so that tetany is impossible; 'all or none' contraction; intrinsic nerve supply and regulatory nerve supply from the outside." The cerebroside content of skeletal muscle increases with growth, whereas that of cardiac muscle decreases.

As a further illustration, the phospholipid component, sphingomyelin,

decreases in concentration in both of these types of muscle during growth. In the lung and kidney, both organs in which a large part of the physiological functions are concerned with the transfer of biochemical substances across semipermeable membranes, the content of sphingomyelin increases during growth. The relatively high content of sphingomyelin found in the intestinal mucosa by Hunter (11) lends support to the speculation that sphingomyelin may be involved in the success of the tissue to accomplish this transfer. Thus, the changes found in the concentration and pattern of the essential lipid components during growth provide information about lipid constituents and tissues other than those studied by Bloor (10). The results of this study extend Bloor's investigation of cholesterol and phospholipid in muscle, and verify his conclusion that "it seems probable that these associations represent, to some extent at least, real functional relations" (10).

#### SUMMARY

The lipid distribution, phospholipid (cephalin, lecithin, and sphingomyelin), free and combined cholesterol, cerebroside, and neutral fat, has been determined in tissues (brain, heart, kidney, lung, testes, liver, thymus, spleen, and skeletal muscle) of rats 15, 45, and 70 days old. All of the tissues increase in essential lipid content during growth. This increase is a reflection of the phospholipid, which is the major component of the essential lipids. The other components of the essential lipid, cerebroside and cholesterol, vary independently, not only with respect to each other but also to the total, during growth of the rat between 15 and 70 days of age.

The fundamental change, quantitatively, in lipid composition during growth, common to all the tissues studied, appears to be an increase in cephalin concentration. Specific changes in the various organs during growth are an increase in cerebroside in testes and skeletal muscle and a decrease in cardiac muscle; a decrease in free cholesterol and sphingomyelin in both skeletal and cardiac muscle; and an increase in sphingomyelin in kidney, lung, and spleen. All essential lipid components except lecithin and cholesterol esters show a marked increase in the brain during growth.

The probable relationships of these changes in lipid composition and pattern among the various tissues to the structural and functional growth of the tissue are discussed.

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# PREPARATION, PROPERTIES, AND PARENTERAL ADMINISTRATION OF TRYPTOPHANE-CONTAINING ACID HYDROLYSATES OF PROTEINS\*

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In a preliminary communication published several years ago (1), there was described a procedure for the preparation of tryptophane-containing acid hydrolysates of proteins. These hydrolysates were found to be suitable for intravenous administration. It has now been possible to complete this investigation.

The problem of parenteral administration of nitrogen derived from protein sources has been adequately reviewed (2-5). It has been demonstrated that experimental animals and humans utilize parenterally administered nitrogen for maintenance of positive nitrogen balance and for plasma protein regeneration (2-15), and satisfactory growth has been reported for rats receiving protein nitrogen solely via the parenteral route (10). The sources of nitrogen employed have generally been products prepared from proteins by enzymatic digestion or by acid hydrolysis. More recently, satisfactory nitrogen balance and plasma protein regeneration have been achieved experimentally by parenteral administration of synthetic mixtures of pure amino acids (8, 12, 14, 15).

Enzymatic digests of proteins contain the original complement of amino acids present in the protein, and, when prepared from a nutritionally adequate protein, do not require amino acid supplements. The chief undesirable feature of enzymatic digests for parenteral use appears to be the crude enzyme materials employed for large scale preparations. Ground, fresh hog pancreas has been used for the commercial preparation of certain enzymatic digests (16), and papain for others (9). While there is no definite evidence that such products contain foreign allergens, some undesirable contaminants present in the enzyme preparation might appear in the final product prepared from the digest.

Complete acid hydrolysates of proteins have been utilized by a number of investigators as a source of parenteral nitrogen (2-5), but require the addition of tryptophane, since this essential amino acid is largely destroyed

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under the usual conditions of hydrolysis. The pure amino acid or a tryptophane-containing protein hydrolysate prepared by alkaline hydrolysis may be added to the acid hydrolysates to replace the tryptophane destroyed by the acid treatment of the protein. The cost of pure tryptophane has previously made this approach to the clinical problem impractical. This objection is now less valid as a consequence of the new syntheses of this amino acid reported recently from two laboratories (17, 18), thus making *dl*-tryptophane more readily available. However, experimental evidence strongly indicates that complete acid hydrolysates, fortified with tryptophane, or synthetic mixtures of amino acids, are not as efficiently utilized for the nitrogen needs of the organism when given by vein as are mixtures of larger molecular weight peptides resulting from partial degradation of proteins (14). The greater rate of loss through the kidneys of moieties of lower molecular weight may contribute to these differences. Also, there may be amino acid aggregates of optimum size for most efficient utilization by the synthetic mechanisms. The latter factor could be particularly significant when the amino acid composition of the protein hydrolysate used for parenteral administration closely approximates the organism protein being replaced.

For these reasons, it seemed desirable to establish conditions of acid hydrolysis of protein which would hydrolyze the protein to smaller fragments that could be given rapidly by vein with no undesirable reactions, and yet cause minimal destruction of tryptophane. Mild acid hydrolysis of proteins afforded the desired solution of the problem. If casein is refluxed with relatively weak concentrations of sulfuric acid for short periods of time, hydrolysates are obtained with varying quantities of free amino nitrogen and of tryptophane. Application of the ninhydrin method (19) to the hydrolysates has also afforded an approximate index of the degree to which free amino acids were liberated under the various hydrolytic conditions. The usefulness of the application of both the nitrous acid and ninhydrin methods of Van Slyke in interpreting the course of protein hydrolysis has been illustrated in studies of the partial hydrolysis of proteins by enzymes (20, 21) and by concentrated hydrochloric acid (22, 23).

The data for casein hydrolysates are presented in Table I and those for hydrolysates of pancreas protein and of pumpkin seed globulin are in Table II. The data show that approximately 50 per cent of the potentially available free amino groups of casein, pancreas protein, and pumpkin seed globulin is liberated by hydrolysis of the proteins with 1.0 *N* sulfuric acid for 6 to 16 hours, or with 2.6 *N* acid for 3 to 6 hours. As might be expected, the extent of hydrolysis of the proteins is dependent on the concentration of acid and the length of the hydrolysis period. It is also to be noted that, under the experimental conditions employed, no great differences in result

were observed when the ratio of acid to protein was 20:1 as compared to 10:1.

It is also evident from the data in Table I that a stronger concentration of sulfuric acid (5.5 N) effects a rapid hydrolysis of casein in a relatively short period of time (6 to 8 hours). At this time approximately 55 per cent

TABLE I  
*Hydrolysis of Casein by Acid*

Acid (ml.) Protein (gm.)	Acid concentration	Hydrolysis period	Amino N in hydro- lysate	Carboxyl N in hydro- lysate	Extent of hydrolysis*	Tryptophane†	
						Per cent in hydro- lysate	Per cent of original
		hrs.	per cent of total N	per cent of total N	per cent		
20:1	8.0 N H <sub>2</sub> SO <sub>4</sub>	24	74.8	88.6	100.0	0.00	0.0
10:1	1.0 " "	3	18.6	5.5	24.8	0.99	87.6
10:1	1.0 " "	6	25.6	12.4	35.4	0.89	78.8
10:1	1.0 " "	16	39.6	29.8	53.0	0.70	62.0
20:1	1.0 " "	16	41.7	31.0	55.7	0.89	78.8
10:1	2.6 " "	3	35.8	17.6	47.7	0.91	80.5
10:1	2.6 " "	6	48.1	32.2	64.2	0.76	67.2
20:1	2.6 " "	6	53.5	38.0	69.0	0.76	67.2
20:1	5.5 " "	3	56.7	43.7	74.5	0.81	71.7
20:1	5.5 " "	6	68.1	63.3	91.0	0.60	53.1
10:1	5.5 " "	6	67.2	62.4	88.0	0.67	59.2
20:1	5.5 " "	8	74.2	88.0	98.0	0.62	54.8
20:1	5.5 " "	24	74.6	88.5	100.0	0.13	11.5
10:1	0.25 " HCl	8	14.9	4.4	20.7	0.94	83.2
10:1	0.25 " "	12	18.7	5.6	25.0	0.85	75.2
10:1	0.25 " "	17	22.7	9.3	30.6	0.80	70.8
10:1	0.25 " "	24	22.4	9.2	29.8	0.73	64.6
10:1	0.25 " "	48	23.0	9.4	28.8	0.51	38.9

\* The extent of hydrolysis is calculated in terms of the ratio of amino nitrogen present in the hydrolysate to the amino nitrogen liberated by complete hydrolysis; i.e., hydrolysis with 8.0 N H<sub>2</sub>SO<sub>4</sub> for 24 hours.

† The sample of casein used contained 1.13 per cent tryptophane and had a total nitrogen value of 13.1 per cent.

of the tryptophane<sup>1</sup> originally present in the protein can be found in the hydrolysate; continuation of the boiling results in rapid tryptophane de-

<sup>1</sup> Aliquots of the acid hydrolysates were completely hydrolyzed with alkali before application of the analytical method for tryptophane. Low tryptophane values will be obtained if the colorimetric method used in this study for tryptophane estimation is applied to partial hydrolysates of proteins. There is an incomplete precipitation of tryptophane-containing peptides by mercuric sulfate, and the chromogenic value of tryptophane in peptides is less than that of the free amino acid.

struction. The early, rapid splitting of peptide bonds of proteins by acid hydrolysis has been observed by a number of investigators (22-25).

The casein used in this investigation was an inexpensive, commercial product whose relatively low nitrogen content (13.1 per cent) indicated the presence of impurities. This product was advisedly chosen, not only because of its availability in quantity at low cost, but also because it afforded an opportunity to study tryptophane preservation under the least favorable conditions; *i.e.*, in the presence of impurities which are known to favor tryptophane destruction. However, it is clear from the data in Tables I

TABLE II

*Hydrolysis of Pancreas Protein and of Pumpkin Seed Globulin by Sulfuric Acid*

	Acid (ml.) Protein (gm.)	Sulfuric acid con- centration	Hydrolysis period	Amino N in hydrol- ysate	Carboxyl N in hydrol- ysate	Extent of hydrol- ysis*	Tryptophan†	
							Per cent in hydrol- ysate	Per cent of original
		%	hrs.	per cent of total N	per cent of total N	per cent		
Pancreas protein	20:1	8.0	24	76.1	84.0	100.0	0.00	0.0
	10:1	1.0	3	24.3	1.8	31.9	1.05	79.0
	10:1	1.0	6	40.0	14.4	52.5	1.00	76.3
	10:1	2.6	3	36.0	11.4	47.3	0.86	66.0
	10:1	2.6	6	44.8	21.6	58.8	0.76	58.1
Pumpkin seed globulin	20:1	8.0	24	81.2	87.8	100.0	0.22	16.5
	10:1	1.0	3	23.3	5.4	28.7	1.14	85.7
	10:1	1.0	6	36.5	11.8	45.0	0.93	69.8
	10:1	2.6	3	35.0	11.0	43.2	0.90	67.6
	10:1	2.6	6	44.6	17.1	55.0	0.85	63.9

\* The extent of hydrolysis is calculated in terms of the ratio of the amino nitrogen present in the hydrolysate to the amino nitrogen liberated by complete hydrolysis.

† The sample of pancreas protein as used contained 14.2 per cent nitrogen and 1.30 per cent tryptophane; the pumpkin seed globulin had 15.1 and 1.33 per cent of nitrogen and tryptophane, respectively.

and II that, in the case of each of the three proteins studied, acid hydrolysates may be obtained which represent a significant degree of protein splitting and yet retain more than 50 and, in some instances, as much as 85 per cent of the tryptophane originally present in the protein.\*

The use of sulfuric acid for hydrolysis necessitates the removal of excess

\* Similar results have also been obtained by mild acid hydrolysis of lyophilized horse serum and dried beef serum. The data in this paper for pancreas protein hydrolysates are in satisfactory agreement with those obtained in the Sharp and Dohme laboratories by L. S. Ciereszko, R. J. Westfall, and E. A. Risley who hydrolyzed pancreas protein under comparable conditions (private communication from Dr. L. Earle Arnow).

acid, conveniently with baryta or calcium hydroxide, before the hydrolysates can be readied for parenteral administration. Large scale hydrolysate preparation therefore involves the inconvenient handling of large quantities of bulky, insoluble sulfate. The desirability of washing the precipitate thoroughly to minimize amino acid loss further lengthens the procedure. The influence of these undesirable features can be lessened somewhat by using the weaker (1.0 N) sulfuric acid. In order to circumvent completely these difficulties, experiments were conducted on the hydrolysis of casein with dilute hydrochloric acid. These hydrolysates were neutralized with sodium hydroxide; the concentration of acid employed was so chosen that, on neutralization and evaporation of the hydrolysate to dryness, a product was obtained with an average salt concentration of 5 to 6 per cent. Therefore, a 20 per cent solution of the final product represented a mixture of approximately 19 per cent of protein degradation products and 1 per cent of salt. These products were well tolerated when given rapidly in 20 per cent aqueous solution by vein to rats, guinea pigs, rabbits, and dogs. The antigenic aspects of the protein have been adequately eliminated, and the weak hydrochloric acid hydrolysates contain a highly significant proportion of the tryptophane originally present in the protein (Table I). It may be pointed out that with the concentration of hydrochloric acid used, maximal protein splitting is achieved at between 12 to 17 hours of hydrolysis. Beyond this period, there is no further hydrolysis apparent, but destruction of tryptophane continues.

As previously indicated, both baryta and calcium hydroxide have been found satisfactory for removal of excess sulfuric acid when this acid has been used for hydrolysis. Experience with baryta has been more extensive; the significant solubility of calcium sulfate leads to the presence of this salt in the final hydrolysates when calcium hydroxide has been used for neutralization. Removal of acid and decolorization of the hydrolysate may be conducted in a single step for convenience. Loss of nitrogen by adsorption on the insoluble sulfate and on the decolorizing carbon may be minimized by thorough washing of the sulfate and by employing norit A.<sup>3</sup> The latter decolorizing carbon has been found the most satisfactory from the standpoint of maximal decolorization of the hydrolysate and minimal nitrogen loss or destruction. This loss has been judged on the basis of the tryptophane and nitrogen contents of acid hydrolysates before and after decolorization, and a comparison of these values with those obtained by analysis of the final, dried partial acid hydrolysates. The tryptophane values obtained on these various products are in satisfactory agreement when norit A has been used to decolorize hydrolysates made from 8 gm. samples of protein. If larger quantities of protein (300 gm.) are hydro-

<sup>3</sup> Eimer and Amend.

lyzed, a loss of 1 to 3 per cent of the hydrolysate nitrogen may occur during removal of the insoluble barium sulfate, even though the latter is washed with liberal quantities of hot water. When the combined filtrates and washings from the barium sulfate are concentrated to dryness *in vacuo*, a residue is obtained which may be ground and sieved. The weights of the dried products thus obtained have varied from 82 to 95 per cent of the weight of protein initially taken for hydrolysis.

The final products are light brown, hygroscopic powders, soluble in 20 per cent concentration in either water or 10 per cent glucose solution. More concentrated aqueous solutions have been prepared; a slight amount of insoluble material, if present, may be removed by filtration. A 20 per cent solution of products prepared from casein has a pH of 4.0 to 5.0. These solutions may be adjusted to pH 7.0 with sodium hydroxide and

TABLE III

*Growth of Rats on Diets Containing Protein or Partial Acid Hydrolysates of Protein*

Type of dietary nitrogen	No. of animals	Average daily weight gain	Average daily food consumption	Weight gain per gm. food eaten
		gm.	gm.	gm.
Casein.....	11	4.8 $\pm$ 0.17*	8.2 $\pm$ 0.16*	0.58 $\pm$ 0.014*
Pumpkin seed globulin.....	8	2.8 $\pm$ 0.32	6.6 $\pm$ 0.56	0.42 $\pm$ 0.016
Pancreas protein.....	19	5.9 $\pm$ 0.12	10.4 $\pm$ 0.24	0.57 $\pm$ 0.009
Casein acid hydrolysate.....	7	1.7 $\pm$ 0.32	4.9 $\pm$ 0.48	0.35 $\pm$ 0.040
Pumpkin seed globulin acid hydrolysate.....	7	2.1 $\pm$ 0.14	5.9 $\pm$ 0.40	0.36 $\pm$ 0.010
Pancreas protein acid hydrolysate.....	7	2.9 $\pm$ 0.18	6.2 $\pm$ 0.41	0.46 $\pm$ 0.017

\* The values in these columns are the means and standard errors.

sterilized by boiling. The sterile solutions have remained clear after standing for 3 months at room temperature. The hydrolysate solutions have been administered rapidly by vein in a number of species (rats, guinea pigs, rabbits, and dogs) without undesirable reactions. Similar tolerance to the hydrolysates has been observed following subcutaneous or intraperitoneal administration of 10 per cent solutions in rats.

Confirmation of the colorimetric analyses showing 50 to 85 per cent of the original tryptophane of the proteins as being present in the hydrolysates was sought in rat growth experiments. Inasmuch as the young rat requires an adequate daily intake of tryptophane, together with the other essential amino acids, as nitrogen for growth, the growth-promoting properties of products prepared by mild acid hydrolysis (2.6 N H<sub>2</sub>SO<sub>4</sub> for 6 hours) were determined. These experiments would also indicate possible other losses of amino acids in the course of preparation of the final hydrolysates. The

growth of young rats on diets whose nitrogen was derived chiefly from partial hydrolysates of casein, pancreas protein, or pumpkin seed globulin was compared to the growth of other animals on diets in which an equivalent amount of nitrogen was fed as unhydrolyzed proteins. The growth data are shown in Table III. Although the average daily weight gain is greater in rats fed diets containing the unhydrolyzed proteins than in animals ingesting the hydrolysate-containing diets, the latter rations were capable of promoting a significant degree of growth in young rats over a 28 day period. These acid hydrolysates, therefore, are capable of promoting growth, but to a somewhat more limited degree when compared with the respective proteins.

#### EXPERIMENTAL

*Proteins Used*—The casein was a commercial product.<sup>4</sup> The pancreas protein was kindly furnished by Dr. L. Earle Arnow of Sharp and Dohme, Inc., and was prepared by a method developed in that laboratory (26). Pumpkin seed globulin was isolated from *Cucurbita moschata*, as described by Vickery, Smith, Hubbell, and Nolan (27).

*Preparation and Treatment of Hydrolysates*—Hydrolysis of proteins was conducted by heating on a sand bath at boiling temperature under a reflux. Initiation of hydrolysis was taken as the time at which boiling of the protein-acid mixture was first apparent. Caprylic alcohol was used to prevent foaming, which was particularly evident when weaker acid concentrations were employed. To obtain the analytical data in Tables I and II, it was found convenient to use 8 gm. samples of protein for each hydrolysis experiment. At the end of the period of hydrolysis, the flask was cooled immediately, surrounded by an ice bath, and brought to pH 7.0 (glass electrode) by the slow addition of a calculated quantity of sodium hydroxide pellets. Vigorous stirring was maintained during the neutralization process. The neutralized hydrolysate was then made to a convenient volume, usually 200 ml., and a small amount of insoluble material removed by filtration with the aid of moderate suction. Suitable aliquots were taken for the analyses; in most instances these aliquots required further dilution before application of the analytical method.

For physiological studies, larger quantities (300 to 1500 gm.) of protein have been subjected to partial hydrolysis with weak acid. At the end of the hydrolysis period, the contents of the boiling flask were diluted with an equal volume of water. The solution was transferred to an enamel pot, brought to a gentle boil, and 1 gm. of norit A added for each 6 gm. of protein taken for hydrolysis. Gentle heating was then continued for 30 minutes and the solution allowed to cool to room temperature.

<sup>4</sup> Lister Brothers, Inc., New York.



The sulfuric acid hydrolysates, after decolorization and without removal of the bone coal, were stirred mechanically and somewhat less than the calculated quantity of powdered, commercial barium hydroxide added slowly with vigorous stirring. Complete precipitation of sulfate was then achieved by careful addition of small quantities of hot, saturated barium hydroxide solution. At this point, small centrifuged aliquots of the mixture showed no turbidity with either a drop of saturated barium hydroxide or a drop of dilute sulfuric acid (1 ml. of concentrated  $\text{H}_2\text{SO}_4$  in 100 ml. of  $\text{H}_2\text{O}$ ).

The mixture was then brought to boiling, stirred with gentle heating for 15 minutes, and the precipitate allowed to settle. The supernatant was filtered rapidly with suction through large Büchner funnels and finally the entire precipitate was transferred to the funnels. Washing of the precipitate was accomplished by returning the contents of the Büchner funnels to enamel pails and, with the aid of vigorous stirring, suspending the precipitate in a volume of water one-third that of the original volume of acid used for hydrolysis of the protein. The suspension was heated for 30 minutes with stirring, and filtered with the aid of suction. The washing procedure was repeated two additional times. The combined filtrate and washings were concentrated to dryness *in vacuo* at  $80^\circ$ . Foaming during distillation was minimized by keeping the volume of liquid in the distillation flasks small and feeding the solution into the flasks at intervals. Combined filtrates and washings were concentrated to dryness. The residues in the distillation flasks were combined and ground in a ball mill; the ground product was then sieved.

In the case of the 0.25 N hydrochloric acid hydrolysates, the mixture, after decolorization with norit A and without filtration, was neutralized by the careful addition of 2 N sodium hydroxide solution to pH 7.0 (glass electrode). After filtration and washing of the bone-coal and sediment, the light yellow filtrate and washings were combined and concentrated to dryness *in vacuo*. The final dry product was obtained by grinding and sieving as described above.

*Analytical Methods*—Total nitrogen was determined by the micro-Kjeldahl method. The amino nitrogen determinations were performed by the Van Slyke nitrous acid method (28) and carboxyl nitrogen analyses by the ninhydrin- $\text{CO}_2$  reaction at pH 2.5 (19). Tryptophane analyses were conducted by the method of Folin and Ciocalteu (29), with 20 ml. aliquots of the acid hydrolysates of 8 gm. samples of proteins. To each 20 ml. aliquot, representing 0.8 gm. of the protein, were added 4 gm. of solid sodium hydroxide, thus giving the 20 per cent alkali concentration proposed by Folin and Ciocalteu (29) for alkaline hydrolysis of proteins. The alkaline aliquot was hydrolyzed under a reflux for 20 to 24 hours; the procedure was then that described by Folin and Ciocalteu (29).

*Rat Growth Experiments*—Male rats 21 days of age and of the Yale strain were used in the growth studies. The animals were kept in individual cages and received, in addition to a basal diet, a daily supplement of 400 mg. of dried yeast<sup>5</sup> and 200 mg. of cod liver oil. The casein basal diet contained casein 21.4, sucrose 15, Crisco 25, salts<sup>6</sup> 4, and starch 34.6 per cent. When a source of protein nitrogen other than casein was used in a basal diet, it was incorporated in the diet in an amount which supplied a quantity of nitrogen equivalent to that present in the basal diet containing 21.4 per cent of commercial casein. The various basal diets, therefore, contained as a source of nitrogen either 18 per cent of pumpkin seed globulin, 22 per cent of pancreas protein, 21.5 per cent of casein hydrolysate, 18 per cent of pumpkin seed globulin hydrolysate, or 22 per cent of pancreas protein hydrolysate.<sup>7</sup> The starch content of each of the basal diets was varied so that the sum of the nitrogen source and the starch in the diet totaled 56 per cent.

Food and water were available *ad libitum*; weighings of the food consumed and of the animals were made twice weekly. Each growth study was conducted for a 28 day period.

#### SUMMARY

Casein, pancreas protein, and pumpkin seed globulin have been hydrolyzed for relatively short periods of time with 1.0, 2.6, and 5.5  $\times$  sulfuric acid. The hydrolysates have been analyzed for their amino nitrogen, carboxyl nitrogen, and tryptophane contents, making possible a correlation of the degree of hydrolysis and tryptophane destruction. Hydrolysis of the proteins with 2.6  $\times$  sulfuric acid for 6 hours liberates approximately 50 per cent of the potentially available total amino nitrogen of the proteins. Tryptophane analyses indicate that under these conditions approximately 65 per cent of the tryptophane originally present in the proteins is not destroyed. Partial acid hydrolysates of proteins with a higher content of tryptophane may be obtained by either shortening the period of hydrolysis or decreasing the sulfuric acid concentration. The presence in the hydrolysates of adequate tryptophane for rat nutrition has been demonstrated by rat growth studies.

Sulfuric acid has been removed from these hydrolysates and final, dry products obtained which, in 20 per cent solution, have been administered rapidly by vein in rats, guinea pigs, rabbits, and dogs with no undesirable

<sup>5</sup> Northwestern Yeast Company, Chicago, Illinois.

<sup>6</sup> Osborne and Mendel salt mixture (30).

<sup>7</sup> The acid hydrolysates used in the growth studies were the final, dried products prepared from the respective proteins by hydrolysis for 6 hours with 2.6  $\times$   $H_2SO_4$ .

reactions. 10 per cent solutions have been given subcutaneously and intraperitoneally in rats.

Data are also presented for the partial hydrolysis of casein with 0.25 N hydrochloric acid. Neutralization with sodium hydroxide leads to final dry products with a salt concentration of 5 to 6 per cent, making it possible to administer 20-per cent solutions of this product by vein.

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# A COLORIMETRIC DETERMINATION OF BLOOD ACETOIN\*

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Most of the methods described for the determination of acetoin and biacetyl are based on the original Lemoigne-van Niel procedure (1, 2), which depends upon the formation of insoluble nickel dimethylglyoxime when biacetyl is treated with hydroxylamine in the presence of a nickel salt; acetoin is similarly determined after oxidation to biacetyl by heating with ferric chloride. In a recent modification Stotz and Raborg (3) have employed a colorimetric determination of the nickel in the precipitate instead of the usual gravimetric analysis, and the sensitivity of the method has thereby been greatly increased. A polarographic method for the determination of acetoin and biacetyl has also been described by Greenberg (4).

The method herein described was developed because none of the previous methods was sufficiently sensitive for our purpose; its chief advantages are simplicity and sensitivity. It is based on the color reaction originally discovered by Voges and Proskauer (5), and later shown by Harden and Norris (6, 7) to be due to the reaction between biacetyl and a guanidino group in the presence of alkali. Attempts to increase the sensitivity of this reaction led to the addition of creatine by O'Meara (8) and  $\alpha$ -naphthol by Barritt (9). While the original Voges-Proskauer reaction could not be developed into a satisfactory quantitative method, the Barritt modification was found to be suitable. In this laboratory it was first tried by W. E. Knox, and a similar method has recently been described by Eggleton *et al.* (10). The chemical reactions involved in the development of the red color have been studied (10-12) but not fully elucidated.

Details of the procedure for color development and the method for determining acetoin in blood are described.

## *Colorimetric Determination of Acetoin and Biacetyl*

### *Reagents—*

0.5 per cent creatine; 1 gm. of creatine dissolved in 200 cc. of water.

5 per cent  $\alpha$ -naphthol; 1 gm. of powdered colorless  $\alpha$ -naphthol (redistilled under nitrogen) dissolved in 20 cc. of 2.5 N NaOH. The solution is prepared *immediately* before using.

*Method—*To 5 cc. of solution containing between 1 and 12  $\gamma$  of acetoin or

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biacetyl are added consecutively 1 cc. of 0.5 per cent creatine and 1 cc. of the 5 per cent  $\alpha$ -naphthol solution. The latter reagent should not be prepared until after the creatine has been added to the test solution, and it is used as soon as the  $\alpha$ -naphthol dissolves. The color is allowed to develop at room temperature for exactly 10 minutes when biacetyl is being determined, and 1 hour for the determination of acetoin. The amount of color is then read in an Evelyn photoelectric colorimeter with a 540 filter; a reagent blank prepared simultaneously with 5 cc. of water is used to adjust the galvanometer to 100, and a standard solution containing 5  $\gamma$  of acetoin or biacetyl can be included in each series of analysis.

The colors obtained by this method were reproducible but somewhat unstable. The maximum color was obtained with biacetyl between 5 and 10 minutes after the addition of the alkaline  $\alpha$ -naphthol; fading was barely perceptible on the galvanometer at 15 minutes, and it amounted to a 10 per cent error at 30 minutes. The color obtained with acetoin was more stable; the maximum intensity was reached in 60 minutes after the addition of the alkaline  $\alpha$ -naphthol; fading could not be detected at 75 minutes, and was barely perceptible at 90 minutes. The absorption spectra of the colors obtained with biacetyl and acetoin were quite similar. The peak of absorption occurred at 530  $m\mu$ , and both curves were relatively flat between 520 and 540  $m\mu$ .

Maximum color was obtained with creatine, but other compounds containing the guanidino group (arginine, creatinine, guanidine carbonate, methylguanidine sulfate) gave similar colors in the presence of biacetyl,  $\alpha$ -naphthol, and alkali.  $\alpha$ -Naphthol could not be replaced advantageously by any one of a large number of substances tested, and it was without effect if added to the test solution of creatine and biacetyl after the alkali.

It was found convenient, but not essential, to dissolve the  $\alpha$ -naphthol in the alkali under a stream of nitrogen, since the reagent became colored readily in air. Only freshly prepared reagent, containing no more than a faint yellow color, was used. Less consistent results were obtained when the  $\alpha$ -naphthol was dissolved in 95 per cent alcohol and added separately from the alkali.

*Standard Curves*—A standard solution of biacetyl containing approximately 1 nig. per cc. was prepared from the fraction boiling sharply at 88°. A similar solution of acetoin was prepared from the crystalline polymer<sup>1</sup> that was equally well purified by thorough washing with anhydrous ether or by fractional distillation. Both standards remained unchanged for several months at 6°, after which the biacetyl solution deteriorated.

Suitable dilutions were prepared from each of these standards, so that 5

<sup>1</sup> The acetoin and biacetyl were obtained from the Lucidol Corporation, Buffalo, New York.

cc. contained from 1 to 12  $\gamma$  of acetoin or biacetyl. Colors were developed in a series of such dilutions and read against the reagent blank in the photoelectric colorimeter after 10 minutes for the biacetyl determination and 1 hour for acetoin. The standard curves for both acetoin and biacetyl, shown in Fig. 1, were linear for amounts of acetoin and biacetyl below 5 to 6  $\gamma$ , but departed slightly from this relationship with larger amounts.

### *Oxidation of Acetoin to Biacetyl*

#### *Reagents—*

$\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$  (solid).

50 per cent  $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ ; 500 gm. of  $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$  dissolved in water and diluted to 1 liter.

10  $\text{N}$   $\text{H}_2\text{SO}_4$ ; 280 cc. of concentrated  $\text{H}_2\text{SO}_4$  diluted to 1 liter.

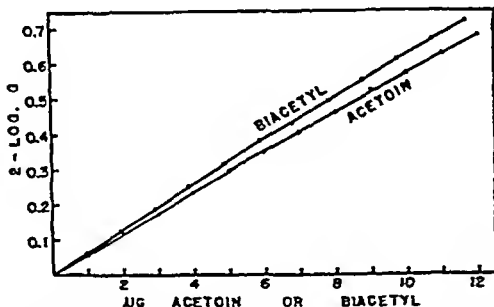


FIG. 1. The relationship of color intensity at 540  $\text{m}\mu$  to the amount of biacetyl or acetoin tested.

When acetoin cannot be determined directly because of interfering substances, a preliminary oxidation to biacetyl and distillation of the latter can be employed; smaller amounts can also be determined by this procedure, since the biacetyl can be concentrated during the distillation. The determination of total acetoin plus biacetyl in mixtures of the two likewise depends upon this preliminary oxidation.

The usual method of carrying out this oxidation with ferric chloride gave 85 to 90 per cent of the theoretical amount of biacetyl; this has been the common experience (13-15). By using a mixture of ferric chloride and ferrous sulfate, as suggested by Knipphorst and Kruisheer (15), the yields were substantially improved, and the following method was adopted.

The oxidizing reagent is prepared by dissolving 10 gm. of  $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$  in 30 cc. of 50 per cent  $\text{FeCl}_3$ . 2 cc. of this solution and 1 cc. of 10  $\text{N}$   $\text{H}_2\text{SO}_4$

are added to 7 cc. of the acetoin-containing solution. The tube is stoppered lightly with a clean rubber stopper and placed in a boiling water bath for 30 seconds to relieve excessive pressure before the stopper is inserted tightly. After heating for 30 minutes in a boiling water bath, the tube is cooled and the contents rinsed into a small distillation flask described by Stotz (16). A glass bead is added and the solution distilled carefully to avoid obtaining any trace of iron salt in the distillate by bumping or spraying. 5 cc. of distillate are collected in a 10 cc. graduated cylinder, and the contents made up to 5.5 or 10 cc., depending upon the amount of biacetyl expected. The color reaction for biacetyl is carried out with 5 cc. of this solution or an aliquot diluted to 5 cc.

TABLE I  
*Oxidation of Acetoin to Biacetyl*

Acetoin oxidized	Biacetyl in aliquot analyzed		Recovery
	Recovered	Theory	
$\gamma$	$\gamma$	$\gamma$	per cent
2	1.82	1.78	102
4	3.66	3.56	103
6	5.22	5.34	98
8	6.86	7.12	96
10	8.72	8.90	98
15	7.20	7.34	98
25	4.83	4.89	99
35	6.67	6.84	98
50	4.60	4.89	94
80	7.50	7.83	96
100	9.35	9.78	96
140	5.30	5.48	97
250	4.87	4.89	100
500	4.83	4.89	99

The recovery of biacetyl from the oxidation of acetoin by this method is shown in Table I. 98 per cent of the theoretical amount was obtained from 2 to 500  $\gamma$  of acetoin; larger amounts were not tested.

*Specificity*—The original Voges-Proskauer reaction is nearly specific for acetoin and biacetyl. The related 5-carbon ketol gives a somewhat similar color, while the 6-carbon ketol gives a light olive-green color within the usual time limit employed (17). In the test herein described, approximately 90  $\gamma$  of methylglyoxal gave as much color as 1  $\gamma$  of biacetyl, while 1 mg. of glyoxal gave no color. 1 mg. of benzil and benzoin in alcohol gave a slight color equivalent to approximately 1  $\gamma$  of biacetyl and 0.5  $\gamma$  of acetoin respectively.

The nickel dimethylglyoxime precipitation is probably more specific for biacetyl than this color reaction. 1.8 mg. of methylglyoxal gave no precipitate in the procedure described by Stotz and Raborg (3). The introduction of  $\alpha$ -naphthol into the reaction also increases the possibility of obtaining colors from unrelated substances that react with the  $\alpha$ -naphthol alone. Such interference, as yet unknown, could readily be detected by omitting the creatine from the test.

### Blood Acetoin

*Method of Determination*—A 1:5 tungstic acid filtrate of blood is prepared by adding 2 volumes of water, 1 volume of blood, 1 volume of 10 per cent

TABLE II  
*Recovery of Acetoin Added to Blood*

Acetoin added	1 cc. blood	5 cc. blood	25 cc. blood
$\gamma$	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1		101	95
2		100	92
3		105	96
5	102	95	92
7	102		89
10	100	93	88
15	97	97	85
25	96	95	
50	96	98	
100	95	101	
250	97	100	
500	95	99	

The acetoin was added to the water with which the blood was laked during the preparation of a protein-free filtrate. The filtrate was analyzed for acetoin by the method described. The percentage recovery includes a correction for the 0.012 to 0.015 mg. per cent of acetoin found in the blood to which the acetoin was added.

sodium tungstate, and 1 volume of  $\frac{2}{3}$  N  $\text{H}_2\text{SO}_4$ ; the precipitate is centrifuged off. An aliquot of the supernatant is added to solid NaCl (1 gm. per 3 cc.) and distilled from a Claisen flask practically to dryness *in vacuo* (150 to 200 mm. of Hg). The distillate is oxidized as previously described by adding approximately 2 cc. of the  $\text{FeCl}_3$ - $\text{FeSO}_4$  mixture and 1 cc. of 10 N  $\text{H}_2\text{SO}_4$  to each 7 cc. of distillate, and heating in a stoppered tube for 30 minutes. The solution is then redistilled, and the biacetyl is collected in the first 5 cc. of distillate and estimated colorimetrically.

The recoveries of added acetoin obtained by this procedure are shown in Table II. For the accurate analysis of the amount of acetoin occurring in normal blood it is necessary to use the maximum amount of filtrate that can



be obtained from a relatively large sample of blood, *e.g.* 15 to 25 cc. When the blood acetoin is elevated slightly, a satisfactory analysis can be run on the filtrate obtained from 5 cc. of blood.

In developing this method for the determination of acetoin in blood, various procedures were tested for the completeness of recovery of added acetoin, and were accordingly developed further or discarded. Direct color development with a tungstic acid or zinc hydroxide filtrate of blood could not be employed because of the appearance of a yellow-brown color during the reaction. When the blood filtrate was oxidized directly with the iron salts previously described and the biacetyl obtained in the distillate was determined colorimetrically, low recoveries were obtained. This was due primarily to the presence of urea in the blood filtrate; urea reacted with the biacetyl, resulting from the oxidation of acetoin, to form a diureide (18, 19). In order to avoid this type of interference by urea (and to a lesser extent by

TABLE III

*Formation of Biacetyl from Glucose by Acid Hydrolysis and Subsequent Oxidation*

Heating prior to oxidation	Biacetyl formed		
	1.2 N H <sub>2</sub> SO <sub>4</sub>	2.2 N H <sub>2</sub> SO <sub>4</sub>	4.2 N H <sub>2</sub> SO <sub>4</sub>
<i>hrs.</i>	$\gamma$	$\gamma$	$\gamma$
0.5	0.44	0.97	3.14
1	0.82	1.87	4.35
2	1.63	3.89	8.30

9 mg. of glucose in approximately 15 cc. of the indicated acid were heated in a stoppered tube in boiling water for the indicated time. After cooling and adding 4 cc. of the FeCl<sub>3</sub>-FeSO<sub>4</sub> solution, the tube was restoppered and heated an additional 30 minutes. The biacetyl was then distilled and determined colorimetrically.

uric acid and allantoin) the acetoin was first distilled from the filtrate and subsequently oxidized to biacetyl for the purpose of concentrating the latter.

*Artifacts in Acetoin Analyses*—During the studies incidental to developing the method for determining blood acetoin, it was observed that relatively large amounts of biacetyl could be obtained from a blood filtrate by subjecting the latter to an acid hydrolysis prior to oxidation with the FeCl<sub>3</sub>-FeSO<sub>4</sub> mixture. Most of this newly formed biacetyl was found to be derived from glucose, and was unrelated to the preexisting blood acetoin. Although the existence of a bound form of acetoin in blood cannot be denied categorically, its existence in appreciable amounts is unlikely, since neither the blood filtrate nor glucose gave rise to any large amount of biacetyl when the oxidizing mixture of FeCl<sub>3</sub>-FeSO<sub>4</sub> was present throughout the hydrolysis.

Table III shows the amount of biacetyl formed from crystalline glucose

under various conditions of acid hydrolysis and subsequent oxidation with the iron salts. The identity of the hiacetyl formed from glucose was established by the following experiments. (a) No color was obtained in the hiacetyl analysis if either creatine or  $\alpha$ -naphthol was omitted from the reaction (the small amounts of biacetyl tested could not give a Voges-Proskauer reaction in the absence of  $\alpha$ -naphthol). (b) 750  $\gamma$  of biacetyl were obtained by heating 1 gm. of glucose in 50 cc. of 10 N  $H_2SO_4$  for 6 hours, diluting, and oxidizing with the  $FeCl_3$ - $FeSO_4$  solution; the biacetyl was concentrated to a 5 cc. volume by two distillations, and a copious red precipitate characteristic of nickel dimethylglyoxime was obtained when this solution was treated with hydroxylamine in the presence of a nickel salt (3). (c) 960  $\gamma$  of biacetyl were similarly obtained after heating 1 gm. of glucose in 200 cc. of 4 N  $H_2SO_4$  for 6½ hours, and the concentrated solution of hiacetyl was allowed to react with 2,4-dinitrophenylhydrazine in 2 N HCl. The precipitate was centrifuged, washed with water and alcohol, and recrystallized from nitrobenzene. The crystals were identical with the bis-2,4-dinitrophenylhydrazone of biacetyl in appearance and melting point (315°), and showed no depression on mixing with an authentic sample.

Pyruvate and lactate gave rise to small amounts of biacetyl when they were treated by the procedure used in oxidizing acetoin to biacetyl. A solution of 100 mg. of sodium pyruvate in 7 cc. of water, 1 cc. of 10 N  $H_2SO_4$ , and 2 cc. of the  $FeCl_3$ - $FeSO_4$  mixture was heated in a stoppered tube for 1 hour and then distilled; 75  $\gamma$  of hiacetyl were obtained in the distillate, and its identity substantiated by conversion to the red precipitate characteristic of nickel dimethylglyoxime. A similar treatment of 100 mg. of lactic acid yielded 2.4  $\gamma$  of biacetyl, while 100 mg. of alanine gave none.

The preliminary distillation of acetoin from the blood filtrate was necessary in order to eliminate interference by substances that combined with biacetyl, as well as those that gave rise to it during the chemical reactions incidental to its determination. We cannot be certain that the small amount of acetoin found normally in blood was not an artifact due to the distillation of some other substance that gave hiacetyl when treated with acid  $FeCl_3$ - $FeSO_4$ . That the chromogenic material obtained from blood was actually hiacetyl was indicated by the absence of color when creatine or  $\alpha$ -naphthol was omitted from the test, and the formation of the characteristic red precipitate when the hiacetyl obtained from 680 cc. of beef blood was treated with hydroxylamine and a nickel salt. Enrichment of the blood with 10 mg. per cent of sodium pyruvate, 10 mg. per cent of acetaldehyde, 300 mg. per cent of glucose, or 200 mg. per cent of alcohol did not alter the amount of hiacetyl obtained in the analysis.

It is probable from these studies that the major part of the acetoin previously identified in normal blood (20) was actually formed from some other

constituent, such as pyruvate, during the chemical reactions involved in the isolation procedure. The relatively large amount of acetoin previously reported (21) in normal blood is undoubtedly an artifact for the same reason.

*Normal Blood Acetoin*—The acetoin concentration in freshly drawn blood from the rat, dog, cat, fasted rabbit, pig, and cow varied from 0.005 to 0.015 mg. per cent, with most of the values falling between 0.007 and 0.012 mg. per cent. Pigeon blood averaged about 0.03 mg. per cent. The concentration of acetoin in beef plasma was approximately 80 per cent of that observed in whole blood; when the acetoin concentration was elevated to 0.365 mg. per cent in rat blood by the administration of acetaldehyde, the plasma contained 0.413 mg. per cent.

#### SUMMARY

A method has been described for the colorimetric determination of acetoin in blood. Possible interference from several constituents of normal blood was described.

The normal blood concentration of acetoin in most of the species studied was approximately 0.01 mg. per cent.

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# THE METABOLISM OF ACETALDEHYDE\*

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It is relatively certain that the first step in the metabolism of ethyl alcohol is an oxidation to acetaldehyde (1). The only known enzyme that will attack alcohol is the alcohol dehydrogenase which converts it to acetaldehyde. This enzyme is located almost exclusively in the liver, and there is abundant evidence from perfusion studies and liver poisoning that the liver is the principal organ in the body capable of the initial oxidation of alcohol. Moreover the blood acetaldehyde is appreciably elevated in some species during alcohol metabolism (2).

The immediate fate of the acetaldehyde so produced is unknown. Studies *in vitro* have shown that there exist in animal tissues at least four or five different enzymes capable of utilizing acetaldehyde as a substrate. These enzymes are the following (3, 4): (1) Xanthine oxidase (Schardinger enzyme), a flavoprotein that not only oxidizes coenzyme I and certain purines but is also capable of converting acetaldehyde to acetic acid with a direct utilization of oxygen. It is located primarily in the liver, but is also found in the red blood cells and milk of some species. Another flavoprotein found in liver, the aldehyde oxidase, is similar to the Schardinger enzyme, but its action is limited to aldehydes. (2) Aldehyde mutase, classically defined as an enzyme that converts 2 moles of acetaldehyde to 1 mole of alcohol and 1 mole of acetic acid. However, since diphosphopyridine nucleotide (coenzyme I) acts as a hydrogen carrier in this reaction, the oxidation-reduction is not limited to a reaction between 2 moles of acetaldehyde, but preferentially takes place with 1 mole of acetaldehyde and 1 mole of another substrate (*e.g.* pyruvate) acting as the hydrogen acceptor. The result of this type of coupled reaction would be the oxidation of acetaldehyde to acetic acid and the reduction of pyruvate to lactate. This enzyme is localized primarily in the liver in animal tissues, but is also found in yeast and bacteria. (3) Aldolase, an enzyme found in muscle and yeast that splits fructose diphosphate into triose phosphates; the reaction is reversible and the enzyme is also capable of recombining aldehydes, such as acetaldehyde, with the dihydroxyacetone phosphate, to form in this case a methyl tetrose phosphate. The possible significance of this enzyme and this type of reaction in the ultimate fate of acetaldehyde

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is obscure. (4) Animal carboxylase, a diphosphothiamine enzyme, widely distributed in animal tissues and abundant in muscles, that condenses acetaldehyde with pyruvate to form acetoin.

Theoretically in the intact animal all of these enzymes could be involved in the metabolism of acetaldehyde, and there is always the possibility that acetaldehyde enters into reactions not yet discovered. Of the known possibilities studied *in vivo* it is improbable that the classical mutase action (conversion of 2 moles of acetaldehyde to 1 mole of alcohol and 1 mole of acetic acid) plays any major rôle in the metabolism of acetaldehyde, since alcohol is not formed during the metabolism of acetaldehyde (5).

The studies herein described were carried out to determine the relative importance of the acetoin pathway in the metabolism of acetaldehyde. Since the xanthine-aldehyde oxidase and aldehyde mutase are found primarily in the liver, while the aldolase and carboxylase are abundant in muscles, the importance of the liver in the metabolism of acetaldehyde was determined following the removal of various organs; it was found that the liver plays a very important rôle in acetaldehyde metabolism. The metabolism of acetaldehyde during thiamine deficiency was also studied since only the acetoin-forming enzyme is known to be a diphosphothiamine protein; the rate of acetaldehyde metabolism was affected only slightly. Therefore acetoin does not appear to be the major product of acetaldehyde metabolism.

## EXPERIMENTAL

### *Methods*

Cats were anesthetized by intraperitoneal injection of 0.7 cc. of dial<sup>1</sup> per kilo, and 1 to 2 cc. of heparin<sup>2</sup> was injected into the exposed femoral vein before a glass cannula was inserted into the carotid artery. Blood samples were removed from the carotid cannula, after a small "wash-out" fraction was discarded and the cannula was attached to a mercury manometer for recording blood pressure. The arterial pressure was maintained above at least 80 mm. of Hg throughout most of the experiments by transfusion with normal cat blood when necessary.

A 1:5 tungstic acid filtrate was prepared immediately from each 5 cc. of blood sample (oxalated), and the precipitate was removed by centrifuging. All manipulations were carried out rapidly in stoppered vessels in an ice bath. Each protein-free filtrate was analyzed for acetaldehyde (2) and acetoin (6). A control blood sample was removed prior to the injection of 2 cc. per kilo of a 2 per cent solution (by weight) of acetaldehyde in 0.9

<sup>1</sup>The dial contained 0.1 gm. of diallylbarbituric acid, 0.4 gm. of urethane, and 0.4 gm. of monoethylurca per cc.

<sup>2</sup>Lilly, 10 mg. of sodium heparin per cc.

per cent saline into the femoral vein over exactly 5 minutes, and additional blood samples were taken at 3, 5, 10, 15, 20, and usually 30 minutes after completing the acetaldehyde injection.

Various abdominal organs were removed by standard surgical procedures (7), and the weight of each animal was corrected for the removed viscera. The operations included nephrectomy, nephro-hepatectomy, and total and partial evisceration. In partial evisceration the stomach, intestines, spleen, and pancreas were removed; the liver and kidneys were also removed in total evisceration. Operations involving removal of the liver were carried out essentially by the method of Markowitz (7), in which that portion of the vena cava passing through the liver was supplanted by a glass cannula. Heparin was used in all experiments to maintain standardized conditions, but was primarily useful in avoiding clotting in those experiments in which a cannula was inserted in the abdominal vena cava.

High levels of blood acetaldehyde were fatal in the absence of artificial respiration, and the latter was supplied when necessary through a tracheal cannula during the first 5 to 10 critical minutes of the experiment. Artificial respiration was used in the studies on eviscerated and hepatectomized animals as well as in normal cats when twice the usual dose of acetaldehyde was administered. Liverless animals were also given hourly 10 cc. per kilo of 10 per cent glucose in saline intravenously.

### *Results of Organ Removal*

The rate of disappearance of the blood acetaldehyde in normal cats and after the removal of various organs is shown in Fig. 1; the corresponding changes in blood acetoin are shown in Fig. 2. These curves represent the average of the results obtained in two normal, two nephrectomized, three nephro-hepatectomized, four totally eviscerated, and two partially eviscerated cats; individual curves within each group were consistently close to the average result shown.

Comparison of these curves (Fig. 1) shows that the rate of acetaldehyde metabolism was appreciably slowed only when the liver was excluded. Removal of the kidneys alone had only a slight effect, while removal of both the kidneys and the liver greatly retarded the rate of disappearance of the blood acetaldehyde. Similarly the effect of partial evisceration was small, even though the portal circulation to the liver was eliminated, while the additional removal of the kidneys and the liver in total evisceration gave the maximum slowing of acetaldehyde metabolism that was observed. A single experiment in a depancreatized cat that had been operated on 11 days previously and deprived of insulin for 72 hours yielded results similar to those obtained in the nephrectomized animals.

The formation and disappearance of blood acetoin (Fig. 2) roughly paralleled the blood acetaldehyde level. In the normal animal the blood

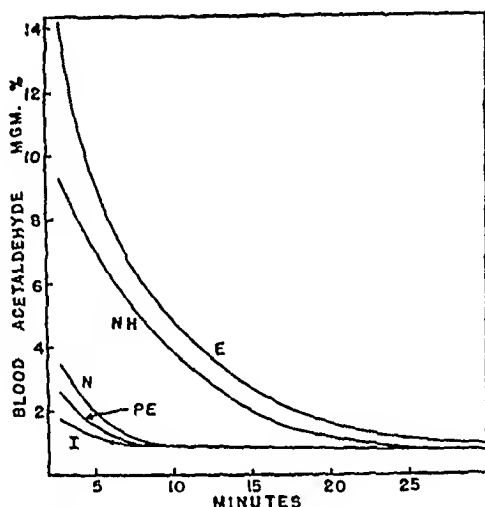


FIG. 1. Blood acetaldehyde disappearance in cats after removal of various organs. 40 mg. of acetaldehyde per kilo were injected intravenously during the 5 minutes immediately preceding 0 time. *I*, intact normal cat; *PE*, partial evisceration; *N*, nephrectomy; *NH*, nephro-hepatectomy; *E*, total evisceration.

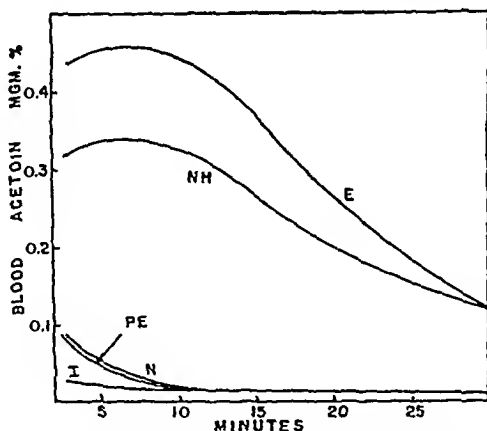


FIG. 2. Blood acetoin changes after acetaldehyde administration to cats deprived of various organs. For explanation of the symbols, see Fig. 1.

acetoin was increased from the control value of 0.015 mg. per cent to about 0.03 mg. per cent at the 3 minute point. With a slightly elevated blood acetaldehyde, as in the nephrectomized and partially eviscerated

animal, the corresponding blood acetoin was 0.08 to 0.09 mg. per cent. The absence of the liver in both the nephro-hepatectomized and totally eviscerated preparation led to a marked and prolonged elevation of the blood acetoin. This could have been due to the prolonged elevation of blood acetaldehyde in these experiments, a slower rate of removal of acetoin in the absence of the liver, or the diversion of a larger proportion of the acetaldehyde through the acetoin pathway.

The effect of an elevated blood acetaldehyde *per se* on the acetoin curve was determined in three normal cats by injecting twice the usual dose,

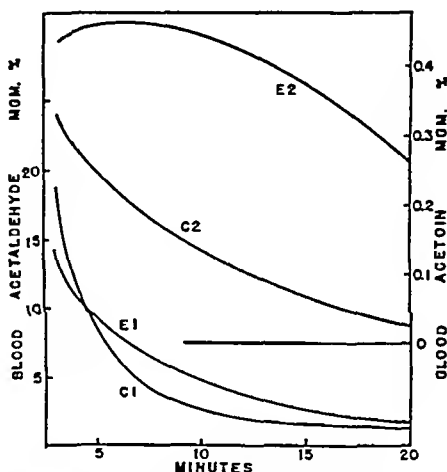


FIG. 3. Blood acetaldehyde and acetoin changes following a large dose of acetaldehyde. Curves C1 and C2, blood acetaldehyde and acetoin respectively in normal cats following the intravenous administration of 80 mg. of acetaldehyde per kilo during the 5 minutes immediately preceding 0 time. Curves E1 and E2, blood acetaldehyde and acetoin respectively in eviscerated cats following the administration of 40 mg. of acetaldehyde per kilo (same curves as are shown in Figs. 1 and 2).

*i.e.* 4 cc. per kilo of 2 per cent acetaldehyde, over the usual 5 minute period. Curves for blood acetaldehyde and acetoin are shown in Fig. 3 for comparison with the results previously observed in eviscerated animals. Since the blood acetaldehyde curves in the two experiments are approximately equivalent, while the acetoin curves are markedly different, it is apparent that the elevated acetoin curves in the hepatectomized animals did not result from an elevated blood acetaldehyde *per se*.

A direct study of the rôle of the liver in acetoin metabolism was made by following the disappearance of acetoin injected intravenously into two



normal, two nephrectomized, and two nephro-hepatectomized cats. The experiments were conducted as previously described except that 0.4 cc. per kilo of 5 per cent *dl*-acetoin instead of the acetaldehyde was injected into the femoral vein over 2 minutes. The average results, illustrated in Fig. 4, indicate a delayed metabolism of acetoin in the absence of the liver, but the effect is not great. It is therefore concluded that in the absence of the liver a larger proportion of administered acetaldehyde is metabolized via the acetoin pathway.

The control blood samples removed after anesthetization and prior to the injection of acetaldehyde or acetoin contained an average of 0.44 mg. per cent of acetaldehyde and 0.015 mg. per cent of acetoin. The latter figure was within the normal range (6) but the normal blood acetaldehyde of 0.02 to 0.05 mg. per cent was elevated by the dial anesthesia. Ether

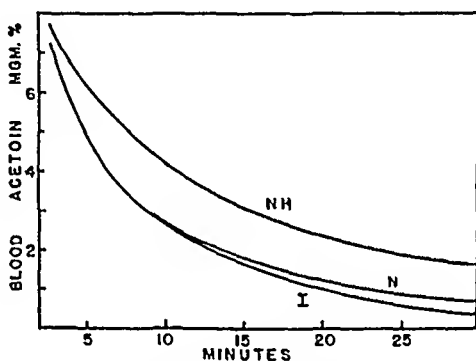


FIG. 4. Disappearance of blood acetoin in the absence of various organs. 20 mg of *dl*-acetoin per kilo were injected intravenously into cats during the 2 minutes immediately preceding 0 time. *I*, intact normal cat; *N*, nephrectomy; *NH*, nephro-hepatectomy.

and nembutal anesthesia did not increase the blood acetaldehyde above 0.03 mg. per cent, and the effect of the dial was attributed to the metabolic formation of acetaldehyde from the urethane and monoethylurea contained therein.

**Excretion of Acetaldehyde**—The acetaldehyde excreted in the expired air during its administration and for 30 minutes afterward was measured in several of the experiments previously described by trapping it in bisulfite and estimating it colorimetrically. The absorption of acetaldehyde in the bisulfite trap was proved to be complete by a test run in which 5 mg. of acetaldehyde were aerated from a solution at 37° for  $\frac{1}{2}$  hour by the artificial respiration apparatus. Normal cats given the usual dose of 40 mg. of acetaldehyde per kilo over 5 minutes excreted about 2.5 per cent in the expired air; totally eviscerated cats excreted about 3.5 per cent. Since the

injected acetaldehyde had largely disappeared from the blood by the end of the experiment, the disappearance curves represent metabolism rather than excretion. The minor difference in acetaldehyde disappearance after nephrectomy indicates a negligible urinary excretion, and Briggs (8) found only a small amount of administered acetaldehyde excreted by the lungs or kidneys.

*Thiamine Deficiency*—Acetaldehyde metabolism was studied in four thiamine-deficient cats (9) prepared by feeding *ad libitum* autoclaved Wilson's dog food<sup>3</sup> supplemented with weekly injections of 1 mg. of pantothenic acid and pyridoxine. A loss of 30 to 40 per cent of body weight occurred during the dietary period of 28 to 42 days before the deficiency symptoms of ataxic gait or complete paralysis appeared. At this point of relatively severe thiamine deficiency the acetaldehyde was injected and studied under the conditions previously described.

The average results obtained for blood acetaldehyde and acetoin in these thiamine experiments are shown in Fig. 5. Comparison with Figs. 1 and 2 shows that acetaldehyde metabolism was not appreciably delayed during thiamine deficiency (*e.g.* equivalent to nephrectomy). The acetoin formation, as reflected by blood levels, was curtailed but not eliminated; blood acetoin rose to about 0.03 mg. per cent, whereas a level of 0.08 to 0.09 mg. per cent would have been predicted from the blood acetaldehyde curve.

Acetaldehyde metabolism was also studied in a thiamine-deficient dog produced by forcibly feeding a synthetic diet (10) for 70 days. At the time the first experiment was run the animal had lost 15 per cent of its body weight and showed the severe thiamine deficiency symptoms of paralysis and convulsive seizures. The dog was anesthetized with nembutal, and 2.5 cc. of 4 per cent acetaldehyde in saline per kilo were injected into a superficial leg vein over 5 minutes. Blood samples were removed from the jugular vein at the usual intervals and treated as previously described. The experiment was repeated after 2 weeks, during which the dog received the same diet supplemented with injections of 10 mg. of thiamine daily; the deficiency symptoms disappeared completely within 6 days of treatment.

Curves for blood acetaldehyde and acetoin in this dog are shown in Fig. 6. During the thiamine deficiency the rate of acetaldehyde disappearance was slowed somewhat, and the acetoin formation, as reflected by blood levels, was practically abolished. Normal curves were obtained after 2 weeks of thiamine treatment. The magnitude of the difference in acetaldehyde metabolism observed in the dog during and after thiamine deficiency was similar to that previously noted between normal and thia-

<sup>3</sup>Wilson's Ideal Dog Food wetted and autoclaved for 6 hours at 15 pounds.

mine-deficient cats. Both series of experiments indicate a minor rôle of the acetoin-forming mechanism in acetaldehyde metabolism, since the former could be seriously curtailed without appreciably affecting the latter.

The effect of inanition was studied in a dog in a similar series of experiments during a 30 day fast. There was a slight but insignificant delay in the rate of acetaldehyde disappearance; however, the blood acetoin did not increase quite as much during fasting, the corresponding values at the 3 minute point being 0.11 and 0.17 mg. per cent of acetoin. The

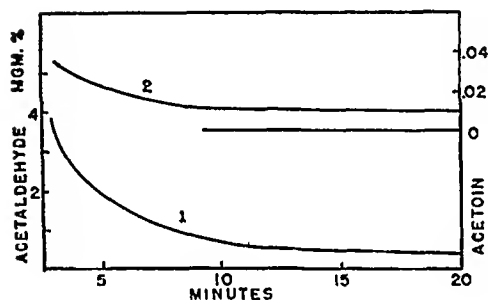


FIG. 5

FIG. 5. Blood acetaldehyde and acetoin changes after acetaldehyde administration to cats during thiamine deficiency. 40 mg. of acetaldehyde per kilo were injected intravenously during the 5 minutes immediately preceding 0 time. Curve 1, blood acetaldehyde; Curve 2, blood acetoin in mg. per cent.

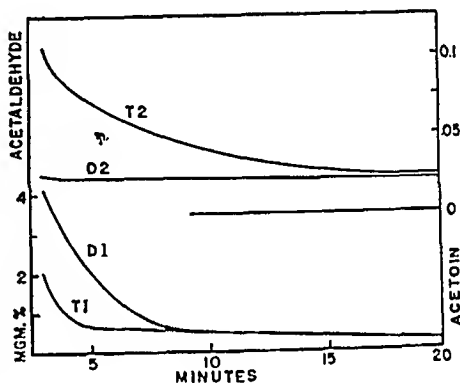


FIG. 6

FIG. 6. Blood acetaldehyde and acetoin changes following acetaldehyde administration to a dog during and after thiamine deficiency. 100 mg. of acetaldehyde per kilo were injected intravenously during the 5 minutes immediately preceding 0 time. Curves D1 and D2, blood acetaldehyde and acetoin curves in mg. per cent during thiamine deficiency; Curves T1 and T2, the same after 2 weeks treatment with thiamine.

effects observed during thiamine deficiency were appreciably greater than those caused by inanition alone.

*Acetoin Formation*—Acetaldehyde was the only substance tested whose administration increased the blood acetoin. This effect was observed in a variety of species, and the average results obtained in unanesthetized rats following the intraperitoneal injection of 200 mg. of acetaldehyde per kilo were almost identical with the curves shown in Fig. 3 for the normal cats. No significant increase in the blood acetoin was observed in rats or dogs following the administration of glucose, insulin, glucose plus insulin, alcohol, pyruvate, or alcohol plus pyruvate.

## DISCUSSION

Acetoin is formed from administered acetaldehyde by a variety of species, but the reaction does not appear to be of major significance in the metabolism of acetaldehyde. The eviscerated preparation metabolizes acetaldehyde very slowly, compared with metabolism in the normal animal; yet the muscles are a good source of the acetoin-forming enzyme. If a major part of the acetaldehyde were converted to acetoin in the intact animal, the muscles would be expected to play an important rôle in this process. Instead it appears that the muscles form large amounts of acetoin from the acetaldehyde only when another pathway, localized primarily in the liver, is excluded.

It is possible that in the intact animal the liver also forms acetoin from the acetaldehyde, and further metabolizes it at a rate that prevents its accumulation. This seems unlikely, since the hepatectomy studies did not indicate an especially important rôle of the liver in acetoin metabolism. Moreover the studies during thiamine deficiency showed that the rate of acetaldehyde metabolism was not appreciably retarded even when the acetoin formation was seriously curtailed.

This combination of results seems fairly conclusive in indicating that acetoin is not a major product of acetaldehyde metabolism in the intact animal. We have no reason to believe that the metabolism of small amounts of acetaldehyde formed continuously during alcohol metabolism would be appreciably different from that observed when acetaldehyde is administered, and it can therefore be assumed that acetoin is not a major product of alcohol metabolism.

The evidence further indicates that the aldolase enzyme is not an important factor in acetaldehyde metabolism, since the principal reaction occurs in the liver. Both of the known enzymes abundant in liver convert acetaldehyde to acetic acid, and in a choice between the mechanism mediated by the flavoprotein or the diphosphopyridine nucleotide enzyme the latter is favored because of the increased blood lactate (11, 12) following acetaldehyde administration. This suggests a coupled oxidation-reduction reaction between acetaldehyde and pyruvate.

## SUMMARY

The disappearance of administered acetaldehyde and the corresponding changes in blood acetoin were studied in cats after nephrectomy, nephro-hepatectomy, and total and partial evisceration. The rate of acetaldehyde metabolism was appreciably slowed only when the liver was removed. The level of blood acetoin roughly paralleled that of blood acetaldehyde.

Similar studies in thiamine-deficient cats and a dog showed that the rate of acetaldehyde metabolism was not appreciably delayed when acetoin formation was seriously curtailed.

The results indicate a major rôle for the liver and a minor rôle for the acetoin-forming mechanism in acetaldehyde metabolism.

No significant increase in the blood acetoin was observed in rats or dogs following the administration of glucose, pyruvate, or alcohol.

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# THE BIOLOGICAL ACTIVITY OF PANTOLACTONE AND PANTOIC ACID

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As far as we are aware, no distinction has been made between the biological activity of pantolactone ( $\alpha, \gamma$ -dihydroxy- $\beta, \beta$ -dimethylbutyrolactone) and pantoic acid or its ion.<sup>1</sup>

In connection with studies on the antagonistic action of pantoyletaurine on salicylate inhibition of *Escherichia coli*<sup>2</sup> we have observed that pantoic acid, or, more properly, the pantoate ion, is more active than pantolactone.

In low concentrations, salicylic acid is thought to inhibit growth of bacteria which synthesize pantothenate (e.g., *Escherichia coli*) by preventing the formation of pantoic acid or pantolactone (2). If such bacteria are supplied with pantothenic acid, pantoate, or pantolactone, the inhibitory effect of salicylate is nullified. Presumably, the pantoate or pantolactone is used to synthesize pantothenic acid. The fact that pantoate is more active than pantolactone in antagonizing salicylate indicates that pantoate is more readily utilized than pantolactone for the synthesis of pantothenic acid. This suggests that the immediate precursor in the biological synthesis of pantothenic acid is pantoic acid or pantoate rather than pantolactone.

## EXPERIMENTAL

A solution of 0.832 per cent ( $6.4 \times 10^{-2}$  M) *d*(-)-pantolactone<sup>3</sup> in 0.1 N sodium hydroxide was hydrolyzed to the pantoate by allowing it to stand at room temperature (29°). The reaction was followed polarimetrically until no further change in rotation was observed. This occurred within an hour. The specific rotation,  $[\alpha]_D^{25} = +13^\circ$ , compared favorably with that calculated from the data of Frost (3), and indicated

<sup>1</sup> After the completion of this manuscript, the article by Sarett and Cheldelin (1) appeared, in which these authors note that pantoic acid is 4 to 5 times more active than pantolactone as a growth factor for *Acetobacter suboxydans*. It seems possible that the conditions which they used to hydrolyze the optically active lactone may have racemized the resulting acid. In this case, the relative activity would be twice that given, or about 9 times, as reported for *Escherichia coli* in the present communication.

<sup>2</sup> Stanly, P. G., and Alverson, C. M., *Science*, in press.

<sup>3</sup> Specific rotation  $[\alpha]_D^{25} = -49^\circ$  (2 per cent solution). We wish to thank the Lederle Laboratories, Inc., for the pantolactone used in this study.

that hydrolysis was apparently complete. The solution was then diluted with phosphate buffer, pH 6.8, and adjustment of the final solution to pH 6.8 was made with small amounts of dilute hydrochloric acid. The final buffer concentration was 0.02 M with respect to phosphate. This solution was assayed for activity simultaneously with a freshly prepared solution of  $8 \times 10^{-3}$  M *d*(-)-pantolactone in 0.02 M phosphate buffer, pH 6.8, after sterilizing by filtration through a sintered glass filter.

The assay consisted of measuring the growth response to various concentrations of pantoate and pantolactone of *Escherichia coli* inhibited by salicylic acid. MacLeod's chemically defined medium (4) containing

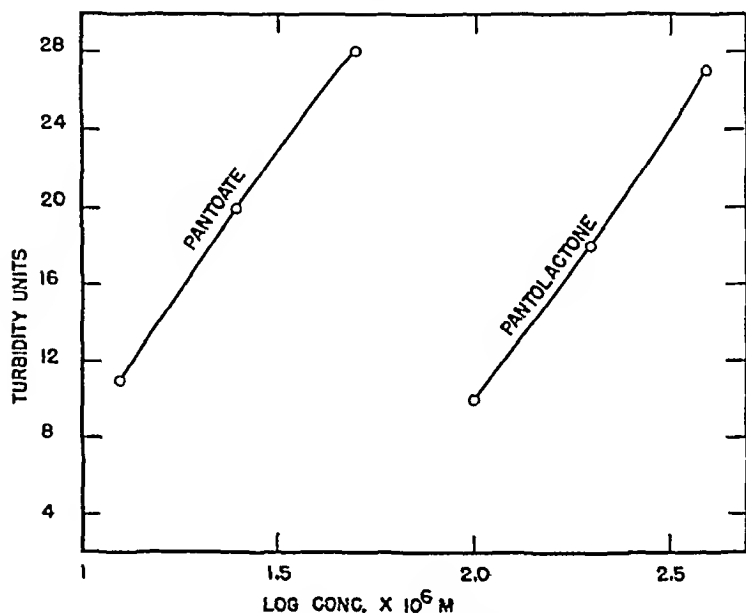


FIG. 1. Relative activity of pantoate and pantolactone. A turbidity of 20 units corresponds to approximately 100 million viable cells per ml.

10 mg. per cent of salicylic acid was used for these experiments. An inoculum of approximately 6 million viable washed cells per ml. (from a 16 hour broth culture) was used and growth at 37° followed turbidimetrically (5).

Fig. 1 shows the relation between the log of the concentration and the turbidity at 8 hours when multiplication was logarithmic. For any given turbidity, the antilog of the log of the concentration of pantolactone minus the log of the concentration of pantoate expresses the relative activity of the two drugs. The results indicate that pantoate is about 9 times more active than pantolactone in antagonizing salicylate inhibition.

It should be noted that this ratio is merely an expression of the relative activity of two solutions prepared in the described manner. It is possible that at least part of the activity in the solution of pantolactone may actually have been due to pantoate. Frost (3) has shown that a rather slow hydrolysis of pantolactone occurs at pH 6.6 and 60°. However, we have not observed any definite increase in biological activity of a solution of pantolactone allowed to incubate at pH 6.8 for 24 hours at 37°. On the other hand, after 24 hours at 37°, a solution of pantolactone at pH 7.8 was found to be 2.5 times as active as it was initially, indicating hydrolysis of about 28 per cent. The hydrolysis was accompanied by a change in the buffer (0.02 M phosphate) from pH 7.8 to 7.4.

#### SUMMARY

Pantoic acid, or, more properly, the pantoate ion, has been shown to be approximately 9 times more active than pantolactone in antagonizing the inhibitory action of salicylic acid on *Escherichia coli*. This suggests that pantoate is more readily utilized than pantolactone by *Escherichia coli* for the synthesis of pantothenic acid. It therefore appears likely that the immediate precursor in the biological synthesis of pantothenic acid is pantoic acid or pantoate, rather than pantolactone.

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# DETERMINATION OF THE DEGREE OF HYDROLYSIS OF PARTIAL ACID HYDROLYSATES OF CASEIN AND FIBRIN

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(Received for publication, July 16, 1945)

White and Elman (1) in 1942 described conditions for the partial acid hydrolysis of proteins under which most of the tryptophane was retained undamaged. These hydrolysates appeared suitable for intravenous injection, as was reported briefly in the same communication. Studies have since been carried out by Risser, Schenck, and Frost (2) in these laboratories which demonstrate that partial acid hydrolysates prepared from casein and fibrin are well utilized by the dog when administered by vein. Of particular interest in regard to the nature of such hydrolysates is the degree of hydrolysis; i.e., the ratio of free amino acids to those in peptide linkage and the relative size of the peptide fragments.

Experiments were made to determine the variation in the degree of hydrolysis of casein and fibrin which would occur at intervals ranging from 2 to 24 hours in boiling 2.8  $\times$  sulfuric acid. Experiments were also carried out to show the effect of rapid hydrolysis in the autoclave at elevated temperature.

## Methods

*Partial Acid Hydrolysis*—Casein (Casein Company of America) and blood fibrin (Armour) were hydrolyzed for 2, 4, 5, 6, or 24 hours in boiling 2.8  $\times$  sulfuric acid. The concentration of protein to acid was 1:5 *W/V*. The sulfuric acid was removed as barium sulfate and the filtrate and washings were treated with decolorizing carbon to yield clear solutions of pH 5.0 to 5.5.

The pressure hydrolysis was carried out with 1.4  $\times$  sulfuric acid, the mixture being stirred in a small glass-lined steam-jacketed tank for 3½ hours at 127°.

*"Complete" Hydrolysis*—A part of the 6 hour partial hydrolysate of each protein was made 8  $\times$  with concentrated sulfuric acid and refluxed for 16 hours to provide the standard for "complete" hydrolysis. Sodium hydroxide was used for neutralization of these hydrolysates before analysis for amino nitrogen and free amino acid nitrogen.

*Total nitrogen* and *ammonia nitrogen* determinations were made on all hydrolysates by micro-Kjeldahl (Pregl).

*Amino nitrogen* was determined by the Van Slyke nitrous acid method (3). Under the conditions of analysis, about 30 per cent of the ammonia in

the hydrolysates was found to be measured as amino nitrogen, as described recently by Irving, Fontaine, and Samuels (4).

*Free amino acid nitrogen* was determined by the ninhydrin method of Van Slyke, Dillon, MacFadyen, and Hamilton (5). The all-glass reaction vessel described by Hamilton and Van Slyke (6) was used and the reaction with ninhydrin was carried out at pH 2.5.

### Results

Values for free amino acid nitrogen in complete hydrolysates of casein and fibrin were consistently found to be about 84 and 87 per cent respectively of the total nitrogen minus ammonia nitrogen. Amino nitrogen values were more variable than the ninhydrin values. Average values were 76 and 82 per cent respectively of the total nitrogen minus the ammonia nitrogen for complete hydrolysates of casein and fibrin. Ammonia nitrogen represented about 10 per cent of the total nitrogen in all hydrolysates, indicating that hydrolysis of amide nitrogen was complete at the first stage of hydrolysis studied.

Table I summarizes the essential data.

### DISCUSSION

From Table I it is seen that a gradual liberation of free amino acids occurs under the conditions of the hydrolysis. The remainder of the amino acids presumably exists in peptide form. It would seem likely that there is a gradual reduction in average peptide size as hydrolysis proceeds, and this is indicated from the following calculations.

Stein, Moore, and Bergmann (7) calculated the average peptide size of partial acid hydrolysates of silk fibroin from the data for total amino nitrogen and free amino acid nitrogen. They stipulated that in order to apply this principle there should be essential agreement between values obtained by the two gasometric methods as applied to complete hydrolysates of the protein in question. They also stated that the non-amino nitrogen of the protein should be low, but there appears to us to be no theoretical reason why this latter criterion need apply. The important limitation in application of the principle to hydrolysates of proteins in general appears to be the errors inherent in the methods; *i.e.*, the overmeasurement of aspartic acid by the ninhydrin method, and the failure of the nitrous acid method to measure proline and hydroxyproline. It appears logical, therefore, that where the approximate content of these amino acids is known the maximum error due to their presence can be predicted, and calculations can be made to delimit the true values.

Ideally, the average peptide length of a partial protein hydrolysate is

equal to the total amino nitrogen present in peptides divided by the peptide amino nitrogen. The first value is obtained by subtracting the free amino acid nitrogen of the partial hydrolysate from the free amino acid nitrogen value for the corresponding complete hydrolysate. The latter value is obtained by subtracting the free amino acid nitrogen from the total amino nitrogen of the partial hydrolysate in question. In the case of the 2 hour casein hydrolysate (Table I), the equation will be  $(84 - 14.2) \div (30.6 - 14.2) = 4.2$ .

The above value, 84 per cent, for the free amino acid nitrogen of the complete casein hydrolysate requires correction due to overmeasurement of aspartic acid. The aspartic acid content of casein is of the order of 6 per

TABLE I  
*Hydrolysis of Casein and Fibrin by 2.8 N Sulfuric Acid*

Protein	Hydrolysis	Amino N	Free amino acid N
	<i>hrs.</i>	<i>per cent*</i>	<i>per cent*</i>
Casein	Complete	76	84
"	2	30.6	14.2
"	4	35.2	16.8
"	5	41.4	22.2
"	6	42.7	23.5
"	24	63.8	48.8
Fibrin	Complete	82	87
"	2	34.2	17.6
"	4	44.5	28.5
"	5	44.6	29.5
"	5	45.4	29.9
"	6	46.4	32.0
"	6	48.8	32.5
"	24	66.0	52.4
"	3 at 127°	34.8	25.2

\* Expressed in per cent of the total nitrogen minus the ammonia nitrogen.

cent, according to the compiled values of Block and Bolling (8). Calculating from the known nitrogen content of aspartic acid and of casein, we find that about 5 per cent of the total nitrogen of casein is aspartic acid nitrogen. Since aspartic acid yields exactly twice the theoretical equivalent of CO<sub>2</sub> in the ninhydrin reaction, we can assume that the value 84 per cent for the complete hydrolysate of casein is about 5 per cent too high. Similarly the proline and hydroxyproline content of casein is reported to be about 8 and 2 per cent, respectively, and this leads to a negative error of about 9 per cent in the nitrous acid method. This is partly compensated for by the fact that a positive error of close to 3 per cent results from the

measurement of 30 per cent of the ammonia of the samples as amino nitrogen. Thus the results of the nitrous acid method as applied to complete hydrolysates of casein are about 6 per cent too low. It will be seen that application of these corrections in Table I gives values for the two methods only a few per cent apart. The correction actually overcompensates for the discrepancy in values between the two methods.

The individual errors in the methods as applied to partial hydrolysates are probably smaller than for the complete hydrolysates; however, since they undoubtedly vary with the degree of hydrolysis, no allowances can be made for them. In any case, it will be seen that maximum corrections that need be made would change the calculation for average peptide length by less than 1 unit and would yield values somewhat lower than those obtained from the data of Table I. Calculation of the data for the progressive degrees of hydrolysis indicates that the average peptide length is at the tetrapeptide stage after about 2 hours, the tripeptide stage at about 6 hours, and the dipeptide stage at 24 hours or less. Thus the data can be used to reflect the relative change in average peptide size within limits, although the precise average peptide size may not yet be known.

The errors in the case of fibrin hydrolysate are of a somewhat different order than those for casein; however, the changes in average peptide size for the progressive stages of hydrolysis appear to be similar to those for casein.

It will be noted that values for the hydrolysate prepared in the autoclave do not appear to agree with the values obtained by the other hydrolysates. Other criteria in this laboratory have indicated differences between hydrolysates prepared in the autoclave and those prepared by refluxing. Rearrangement and ring formation in peptides might be expected to occur under pressure at temperatures of 120° and above, according to the early findings of Abderhalden and Komm (9). Such reactions would probably lead to unpredictable analytical values and would conflict with the above hypothesis.

It is interesting to note that a considerable part of the hydrolysis took place in the first 2 hours and that hydrolysis was relatively slow thereafter. With Chibnall's (10) estimate of the weight of amino acids obtained from hydrolysis of 100 gm. of casein, *i.e.* 115.4 gm. of amino acids and an average equivalent weight of 133 for the amino acids of casein, it is possible to estimate that complete hydrolysis of 100 gm. of casein gives rise to 0.87 equivalent of amino acids. Since only 1.4 equivalents of sulfuric acid are used in the hydrolysis per 100 gm. of casein, the effective acid concentration decreased rapidly as the ampholyte concentration increased. This factor probably contributes to the decrease in the rate of hydrolysis with increasing time of hydrolysis.

2, 4, 5, and 6 hour hydrolysates of casein and fibrin have all been used successfully intravenously in nitrogen balance studies in dogs in this laboratory. There has not appeared to be much choice between them with regard to their ability to maintain nitrogen balance. It has been apparent for some time that peptides of the type occurring in such partial acid hydrolysates are well utilized on injection. Just how large these peptides may be and still be utilized is not known.

#### SUMMARY

Hydrolysates of casein and fibrin were prepared by hydrolyzing 20 per cent solutions of the proteins for 2, 4, 5, 6 and 24 hours in 2.8 N sulfuric acid. Variations in the degree of hydrolysis were determined by the Van Slyke gasometric methods for free amino acid and total amino nitrogen.

We are indebted to Mr. Everett Doede for nitrogen determinations and for many check determinations of carboxyl N and total  $\text{NH}_2\text{-N}$ ; also to Mr. Carl Nielsen and Dr. E. H. Volwiler for encouragement and help in carrying out the project.

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# THE ISOLATION OF $\alpha$ -GLYCERYLPHOSPHORYLCHOLINE FROM INCUBATED BEEF PANCREAS: ITS SIGNIFICANCE FOR THE INTERMEDIARY METABOLISM OF LECITHIN\*

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(Received for publication, July 30, 1945)

The structure of the phosphatides suggests the assumption that their enzymatic hydrolysis represents a stepwise degradation leading to the intermediary formation of simpler esters of phosphoric acid prior to the liberation of inorganic phosphate. Since the chemical nature of these intermediary products in mammalian tissues is as yet unknown, an investigation of the problem was undertaken.

At the outset of our work it became clear that our original plan of studying the phosphorus-containing products formed during the action of purified enzymes on pure phosphatides had to be modified. It was found that only fresh suspensions of some tissues were sufficiently active to permit the isolation of products of the enzymatic hydrolysis of phosphatides. Such suspensions contained considerable amounts of phospholipids which were rapidly hydrolyzed during the incubation, but the addition of pure phosphatides enhanced the action of the phospholipid-splitting enzymes only to a relatively small extent.

It was therefore decided to study the fate of the preformed phosphatides during a short incubation of tissue suspensions. This method of approach entailed considerable difficulties. It precluded any attempt to separate the phospholipid-splitting enzymes from the phosphatases which hydrolyzed the intermediary phosphoric esters and thereby interfered with their accumulation. Moreover, it complicated the detection and isolation of such intermediary esters by the necessity of separating them from the large amount of phosphorus compounds originating from other enzyme reactions such as glycolysis or the hydrolysis of nucleic acids.

In the experimental part, it will be demonstrated that the decrease of the phospholipids during the incubation of mammalian tissues is accompanied by the appearance of an acid-soluble phosphorus fraction which can be separated from the bulk of other acid-soluble P compounds by its solubility in alcohol. The amount of this fraction was always smaller than that of the phospholipids that had disappeared. The isolation of a diester of

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phosphoric acid, namely glycerylphosphorylcholine, from this fraction will be described. The structure of this diester in itself suggests its genetic relationship to lecithin. The quantitative correlation of the amounts of the alcohol-soluble, petroleum ether-insoluble P fraction formed to the decrease of the phospholipids strongly supports the assumption that glycerylphosphorylcholine is formed from lecithin by the action of specific "lecitholipases" which are not identical with the fat-splitting pancreas lipase, since active solutions of the latter enzyme are without effect on lecithin.

A similar enzyme system was found in rice bran and in *Aspergillus oryzae* by Contardi and Ercoli (1) in 1935. When these authors incubated lysolecithin for 9 days with purified extracts of rice bran, which they designated as "lecithinase B," they observed that a part of the phosphatide phosphorus became water-soluble without the liberation of inorganic P or choline. The water-soluble P fraction obtained after the incubation did not contain soaps or organically bound fatty acids. These observations were interpreted by Contardi and Ercoli as evidence for the formation of glycerylphosphorylcholine. The product was not isolated in pure form by the Italian authors, but they described its solubility in alcohol and its precipitation from alcoholic solutions by cadmium chloride. In addition, they found that the cadmium chloride compound was very easily soluble in water. Since these findings agree with our own observations on the pure product obtained by us from incubated pancreas, the interpretation given by the Italian authors to the effect of rice bran extracts on lysolecithin appears to be justified.

If the enzymatic liberation of both fatty acid radicals from lecithin in pancreas represents a simple hydrolytic cleavage, the assumption of an intermediary formation of lysolecithin is theoretically unavoidable. This important problem has not been studied in our present investigation. Belfanti and Arnaudi (2) claimed to have demonstrated the formation of lysolecithin after the incubation of lecithin with extracts from pancreas and salivary glands. Their conclusions, however, are not based on the isolation of pure lysolecithin from the incubated material. Nikuni (3) reported the isolation of lysolecithin from emulsions of egg lecithin after the incubation with pancreas extracts.

#### EXPERIMENTAL

*Determination of Lipid P*—The minced tissue (usually 0.5 gm.) was suspended in 2 cc. of water. The suspension was neutralized with a 0.1 N solution of sodium hydroxide against phenolphthalein and transferred into an Erlenmeyer flask containing approximately 40 volumes (100 to 150 cc.) of a mixture of 3 parts of absolute alcohol and 1 part of ether; the mixture

was boiled for a few minutes on a steam bath and filtered into a round bottom flask. The residue on the filter was washed thoroughly with the alcohol-ether mixture. The filtrate was concentrated to dryness under reduced pressure and the residue reextracted with petroleum ether. The petroleum ether extract was filtered and the residue well washed with petroleum ether. In the combined filtrate and washings the total phosphorus was determined according to the method of Fiske and Subbarow (4).

*Phospholipid-Splitting Activities of Various Mammalian Tissues*—Table I demonstrates the phospholipid-splitting activities of various mammalian organs. The technique used was the same in all experiments, so that the results are quantitatively comparable. The tissues were minced with scissors and two samples of 0.5 gm. each weighed on cover-slips. The

TABLE I

*Phospholipid-Splitting Activities of Tissue*

The results represent mg. of P per 100 gm. of fresh tissue.

Organ	Phospholipid P		Petroleum ether-insoluble alcohol-soluble P	
	Initial value	After incubation	Initial value	After incubation
Striated muscle (rat).....	44.8	32.4	8.8	6.8
Liver (rat).....	143.0	127.0	9.0	9.6
Spleen " .....	57.0	41.7	6.8	14.3
Kidney " .....	133.5	114.5	3.2	8.0
Stomach (rat).....	49.6	34.0	3.2	10.6
Small intestines (rat).....	52.0	0	1.5	26.0
Pancreas (beef).....	100.0	40.0	7.8	40.0

material with the cover-slip was then placed in wide test-tubes which contained 2 cc. of water. One of the samples was neutralized against phenolphthalein<sup>1</sup> and then immediately transferred to a 500 cc. Erlenmeyer flask containing 100 cc. of the alcohol-ether mixture. The other was incubated for 3 hours at 37°. Subsequently, the suspension was neutralized against phenolphthalein and extracted with the alcohol-ether mixture.

It can be seen that, during the incubation of the mucosa of the digestive system and of pancreas, a large part of the phospholipids disappears, while relatively small decreases of the phospholipid contents were observed in the other investigated tissues. The large relative and absolute decreases of the phospholipids in the incubated tissues of the digestive system offered favorable conditions for the investigation of the hydrolysis products.

<sup>1</sup> The neutralization is necessary for the purpose of establishing comparable conditions for the extraction of the fresh and the incubated tissues.

An example of the quantitative distribution of the phosphorus fractions in non-incubated and incubated tissues is given in Table II. The principal results of this experiment are representative of the behavior of all investigated tissues of the digestive system; stomach mucosa, however, appeared to be particularly suitable for quantitative studies of the metabolism of the phospholipids because it contains relatively smaller amounts of the other phosphorus fractions than the various other tissues; in addition, its phosphatase content is low.

The data of Table II demonstrate that the decrease of the phospholipids during the incubation is accompanied by an increase of the total acid-soluble P fraction. This increase is much higher than the decrease of the amount of lipid phosphorus, and thus originates at least in part from the breakdown of insoluble P fractions other than phosphatides. The amount of acid-insoluble P remaining after the incubation is so small that the possibility of the transformation of lipid P into acid-insoluble P compounds is

TABLE II

*Quantitative Distribution of Phosphorus Fractions in Non-Incubated and Incubated Rat Stomach*

The results represent mg. of P per 100 gm. of fresh tissues. The total P is 98.

Treatment	Lipid P	Acid-soluble P	Inorganic P	Protein P	Organic acid-soluble P
Fresh.....	34.8	36.6	16.8	26.6	19.8
Incubated.....	11.3	80.0	39.6	6.7	40.4

excluded. Thus, it must be concluded that the phospholipids are transformed into acid-soluble P compounds during the incubation.

Some information regarding the nature of these acid-soluble breakdown products of the phosphatides was obtained when the phosphorus compounds of fresh and incubated tissues were fractionated further. It was found that in fresh tissues almost the total amount of alcohol-soluble phosphorus compounds was soluble in petroleum ether, but insoluble in aqueous trichloroacetic acid. Incubated tissues of the digestive system, however, contained considerable amounts of alcohol-soluble phosphorus compounds which were insoluble in petroleum ether, but soluble in aqueous trichloroacetic acid. The amounts of this fraction (designated Fraction X) in some fresh and incubated tissues are reported in the last two columns of Table I.

The figures there reported were obtained by means of the following technique. The phospholipids were extracted with petroleum ether according to the method previously described. The petroleum ether extract was filtered and the extraction flask was thoroughly rinsed with petroleum

ether, which was then used for washing the filter. After several washings, the filter was replaced in the extraction flask. The residue of the petroleum ether extract together with the filter was extracted with a boiling mixture of 3 parts of alcohol and 1 part of ether. In the filtered extract the total P was determined.

It can be seen that the amounts of Fraction X increase in almost all tissues during the incubation. In those tissues, however, in which a high phospholipid-splitting activity was observed, much larger amounts of Fraction X were formed than in tissues in which the changes of the phosphatide P during the incubation were relatively small.

It appears unlikely that acid-soluble P compounds which cannot be extracted by alcohol from fresh tissues would become alcohol-soluble or would be hydrolyzed to alcohol-soluble phosphoric esters during the incubation. It is likewise improbable that the nucleic acids would be split into alcohol-soluble phosphoric acid esters. There is a possibility that the phosphatides in the petroleum ether extracts of fresh tissues are accompanied by other phosphorus compounds which are soluble in petroleum ether only in the presence of phosphatides; after the incubation, these substances would lose their solubility in petroleum ether because of the disappearance of the phosphatides, but would still be soluble in alcohol. Such an assumption, however, could only explain increases of the Fraction X amounting to much smaller values than those of the hydrolyzed phospholipids. In most of the experiments on stomach, small intestines, and pancreas, the increase of the Fraction X accounted for approximately 50 per cent of the decrease of the phospholipids. Hence, it is probable that Fraction X consists mainly of phosphoric acid esters originating from the enzymatic breakdown of the phospholipids. Some properties of this fraction were studied in preliminary experiments with samples prepared from several batches of twenty-five rat stomachs. It was found that the extracts of Fraction X did not contain measurable amounts of inorganic phosphate. Fraction X in concentrated aqueous solution gave no precipitate with uranyl acetate, barium acetate, lead acetate (neutral or basic), mercuric acetate, or silver acetate. It was precipitated from concentrated alcoholic solution by cadmium chloride, but not by barium acetate or barium hydroxide.

This behavior is different from that of the known components of the acid-soluble P fraction such as hexose phosphates or nucleotides and suggests that Fraction X represents a distinct group of organic P compounds.

*Isolation of Phosphorus-Containing Hydrolysis Product of Lecithin from Incubated Beef Pancreas*—Beef pancreas was used as the material for isolation experiments. As shown in Table I, the characteristic increase of the alcohol-soluble, petroleum ether-insoluble P fraction during the incubation

of beef pancreas is very marked. Since the observations discussed in the preceding section made it appear probable that this fraction consisted mainly of breakdown products of phosphatides, we undertook its preparative isolation and chemical identification.

For obvious reasons, the isolation procedure followed in its first steps as closely as possible the technique adopted in the small scale experiments on rat stomach. This was particularly important for the extraction of the phosphorus compounds from the tissue. It will be remembered that the extraction with hot alcohol offered the simplest possibility of separating Fraction X from the bulk of the other acid-soluble phosphorus compounds. This effect, however, is achieved only if the extraction of the incubated tissue suspension is carried out with approximately 50 volumes of 95 per cent alcohol. It is obvious that the use of such an excess of hot alcohol is almost impracticable for isolation experiments on large amounts of tissue suspensions. On the other hand, the preliminary dehydration of the tissue by other organic solvents was not feasible because mixtures of these solvents with water extract considerable amounts of phosphorus compounds from the tissues. After various trials aimed at the adaptation of the wet lipid extraction for preparative purposes, we finally chose the following procedure for the preparation of Fraction X.

5 pounds of minced beef pancreas were mixed with an equal volume of water and incubated at 37° for 4 hours. The mixture was then acidified to pH 4.7 by addition of acetic acid and quickly heated to 60° in a water bath. The suspension was filtered on a Büchner funnel while still hot. The filtrate was left overnight in the refrigerator and separated from the solidified fats by filtration on a Büchner funnel.

The clear yellow filtrate was brought to pH 6.5 and concentrated to approximately 300 cc. under reduced pressure. It was then heated on a boiling water bath and poured into 1500 cc. of boiling absolute alcohol. After filtration on a Büchner funnel, the filtrate was again concentrated under reduced pressure to a much smaller volume than before (but not to a syrup) and precipitated with an excess of hot absolute alcohol. The concentrations and precipitations with alcohol were repeated until the liquid could be concentrated to a few cubic centimeters without giving a precipitate on addition of an excess of alcohol. *It was found to be essential to continue the concentrations and precipitations to this point.* Otherwise, the contaminations (chiefly breakdown products of proteins) left in the solution by an incomplete precipitation with alcohol could not be removed in the later stages of the preparation. The alcoholic solution contained approximately 400 mg. of organic P, but was practically free of inorganic phosphate.

The solution was concentrated to a syrup and the concentrate taken up in approximately 50 cc. of water. An aqueous, concentrated solution of

mercuric acetate was added until the precipitation was complete. After addition of 5 volumes of alcohol the precipitate was filtered over a thin layer of Hyflo filter aid on a Büchner funnel. The precipitate was washed with alcohol and the mercuric ions were removed from the clear filtrate by means of hydrogen sulfide. Approximately 80 per cent of the organic P remained in the filtrate from the mercuric acetate precipitation.

The filtrate was concentrated under reduced pressure to a small volume. On addition of 10 volumes of acetone a precipitate formed which consisted partially of an oil, partially of flocculent material. The oily part of the precipitate contained the total amount of the phosphorus. Immediately after the addition of acetone, the suspension was transferred to a separatory funnel. The oil sedimented very soon and was separated as completely as possible from the supernatant and from the flocculent material which settled on top of the oily layer.

When a small sample of the oil was refluxed with *N* hydrochloric acid for 3 hours, it was found that no inorganic phosphate was formed, but that choline appeared in the hydrolysate in amounts equivalent to those of the total phosphorus. A glycerol determination on the oil according to the method of Blix (5) revealed the presence of an amount approximately equivalent to that of the phosphorus. As no fatty acids could be detected after acid or alkaline hydrolysis of the material, the presence of lecithin was excluded.

The equivalence of phosphorus and bound choline (no free choline was found in the oil) suggested the assumption that the phosphorus was present in the form of a choline ester. This ester, however, could not be identical with phosphorylcholine, since the hydrolysis resulted in the liberation of choline without the formation of inorganic phosphate. The presence of an equivalent amount of glycerol made it probable that the compound had to be considered as a diester of phosphoric acid with choline and glycerol. The total amount of nitrogen in the acetone-insoluble oil was, however, much higher than that calculated from the results of the choline determinations. The material gave a strongly positive ninhydrin test and contained considerable amounts of leucines.

For the further purification, the phosphorus-containing component was precipitated as a reineckate by the addition of excess of a cold saturated alcoholic solution of ammonium reineckate to a solution of the oil in 25 to 50 cc. of alcohol. (In aqueous solution, no precipitate was obtained on addition of ammonium reineckate.) A granular, light pink, microcrystalline precipitate formed instantaneously. It contained approximately 50 per cent of the phosphorus present. The reineckate was filtered on a Büchner funnel. Since it was rather soluble in alcohol, this solvent was not used for washing. The powdery precipitate was suspended instead in 500 cc. of

acetone and stirred vigorously for 5 minutes. During this treatment, the reineckate partly decomposed and the precipitate assumed a somewhat sticky consistency, but the phosphorus remained quantitatively in the precipitate.

The Reinecke compound was suspended in approximately 150 cc. of water, filtered from a considerable insoluble residue (all the phosphorus was now in the filtrate), and decomposed by adding a hot saturated solution of silver sulfate and some solid silver sulfate. After being mechanically shaken for 10 minutes, the suspension was filtered on a Büchner funnel. The colorless filtrate contained approximately 100 to 150 mg. of organic P. The N:P ratio in the clear colorless filtrate was at this stage approximately 1.5:1. The solution was freed from silver and sulfate ions by hydrogen sulfide in the presence of freshly prepared barium carbonate.

Since the ratio of choline to P in the solution was 1:1, it was certain that the preparation contained even at this stage other nitrogen-containing substances besides choline. The removal of these contaminations proved to be very difficult. After many unsuccessful attempts with various alkalioid reagents, we found that this task could be accomplished by the use of ion exchangers.

After the removal of the hydrogen sulfide by aeration, the solution was shaken for 1 hour with 20 gm. of Amberlite IR-100 for the removal of nitrogenous contaminants and small amounts of barium. The filtrate from the Amberlite contained approximately 100 mg. of organic P and bound choline as practically the only nitrogen-containing substance. The N:P ratio was 1.03:1. The reaction of the filtrate was slightly alkaline.

When the solution was evaporated under reduced pressure, the substance remained as an almost colorless oil. It could be obtained in an analyzable form as a cadmium chloride compound. The oil was dissolved in 50 cc. of hot alcohol. After cooling, a saturated alcoholic solution of cadmium chloride was added until the precipitation was complete. The cadmium compound was filtered on a Büchner funnel, washed with an alcoholic solution of cadmium chloride<sup>2</sup> and with ether, and dried. For the analyses it was dried at 76° in a vacuum dryer.<sup>3</sup>

C 15.27, 15.18; H 3.42, 3.37; N 2.42; P 5.27; Cl 19.52; Cd 35.05

From these figures the composition of the cadmium and chloride-free moiety of the compound could be calculated.

<sup>2</sup> A solution of the precipitant was used for the washing in order to avoid losses due to the considerable solubility of the cadmium chloride compound in alcohol.

<sup>3</sup> Dr. Carl Tiedeke, to whom we are indebted for the microdetermination of C and H, sent us the results with the comment that the cadmium compound was difficult to analyze and that a high degree of accuracy could not be expected with regard to C and H determinations.

$C_8H_{12}NPO_7$ . Calculated. C 34.90, H 8.05, N 5.27, P 11.27  
 Found. " 33.51, " 7.47, " 5.33, " 11.37

*Glycerol Determinations*—Determinations of glycerol according to the method of Blix (5) were carried out on all samples of the final product. The results obtained by this method were frequently checked by control determinations on known amounts of sodium glycerophosphate. These corresponded to yields ranging from 75 to 85 per cent of the theoretical values. This is probably due to the fact that hydriodic acid of the quality required according to the directions of Blix is not available at present. We found, however, that the results of duplicate analyses with the same batch of hydriodic acid agreed within 5 per cent, even if the amounts of glycerophosphate were varied over a wide range. We therefore adopted it as a routine to carry out a control determination on a known amount of sodium glycerophosphate simultaneously with each determination on the diester. The glycerol determination on the same sample of the diester which was used for the elementary analysis reported above gave the results

TABLE III  
*Determination of Glycerol*

Substance	P	Glycerol	Ratio, P to glycerol	Ratio, P to glycerol (corrected)
	mg.	mg.		
Sodium glycerophosphate . .	0.118	0.280	0.802	1.00
Diester. ....	0.162	0.399	0.829	1.03

shown in Table III. The fourth column contains the values for the molecular ratio between glycerol and phosphoric acid, as calculated from the experimental data. The corrected values of this ratio (last column) have been computed from the figures of the fourth column on the assumption that the true ratio for glycerophosphoric acid is 1:1.

*Products of Hydrolysis.* *Choline*—50 cc. of the solution obtained after the treatment with Amberlite were refluxed for 3 hours with 5 cc. of 5 N hydrochloric acid, cooled, and neutralized against Congo red paper, and an excess of an acidified solution of ammonium reineckate was added. A precipitate of glittering crystals appeared immediately. It was filtered after several hours standing in the refrigerator, washed with cold alcohol, and dried at room temperature in an evacuated desiccator.

Found, 47.30 mg. choline reineckate corresponding to 13.60 mg. choline in 50 cc. solution

Total P in 0.5 cc. solution, 0.335 mg.

Molecular ratio, choline to P = 1.04:1



The aqueous solution of the substance gave no precipitate with Reinecke acid before hydrolysis.

*Glycerophosphoric Acid*—400 mg. of the cadmium compound were dissolved in water and decomposed by hydrogen sulfide. After removal of the hydrogen sulfide, the filtrate was refluxed for 3 hours with 0.1 volume of 5 N hydrochloric acid.

The hydrolysate was then treated according to the procedure described by Folch (6) for the isolation of glycerophosphoric acid from cephalin hydrolysates.

65 per cent of the phosphorus contained in the cadmium compound could be recovered in the form of a barium salt with a phosphorus content of 9.52

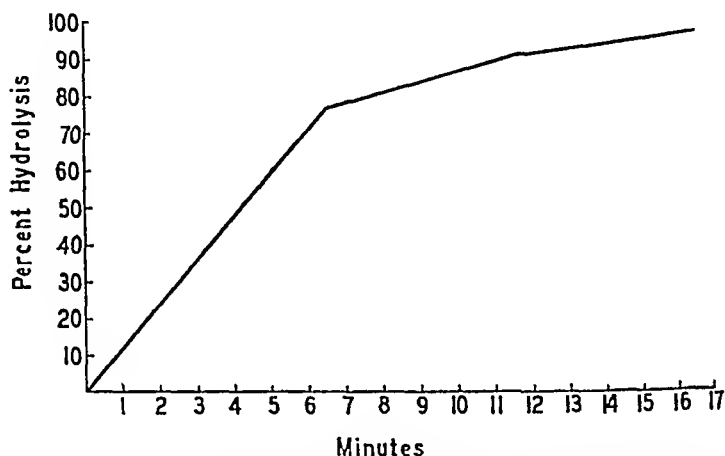


FIG. 1. Hydrolysis of glycerylphosphorylcholine (concentration, 2.06 mM) by N hydrochloric acid at 97°.  $k$  ( $\log_{10}$ ) =  $9.6 \times 10^{-3}$ ,  $9.9 \times 10^{-3}$ ,  $9.2 \times 10^{-3}$ ;  $k$  ( $\log_{10}$ ) (N HCl; 100°) for phosphorylcholine =  $1.3 \times 10^{-4}$  (6).

per cent (calculated for  $C_3H_7O_6P\text{Ba}$ , 10.1 per cent). The barium salt was free of nitrogen.

The glycerol determination was as follows:

	Glycerol	P	Molecular ratio, glycerol to P
	mg.	mg.	
Barium glycerophosphate.....	0.453	0.189	0.80
Sodium " (control).	0.583	0.236	0.83

The rate of the hydrolytic formation of choline was very rapid in N hydrochloric acid at 97° (Fig. 1). The hydrolysis under these conditions liberates approximately two-thirds of the total choline within 5 minutes

and is practically complete within 15 minutes. This behavior is in sharp contrast to the great stability of phosphorylcholine (7). On the other hand, it is known that the ester linkage of choline in lecithin is very labile towards acids. No inorganic phosphate appeared during the hydrolysis of the ester with *N* hydrochloric or sulfuric acid under conditions which resulted in the quantitative liberation of the choline group.

*Reaction of Diester with Sodium Periodate (8)*—10 cc. of a diester solution containing 0.81 mg. of diester P were mixed with 5 cc. of water, 5 cc. of 0.1 *N* acetate buffer of pH 5.3, and 5 cc. of a 0.0038 solution of sodium periodate. A control mixture in which the diester solution was replaced by water was treated in the same way. After 5, 15, 20, and 90 minutes the amount of periodate was determined in 5 cc. aliquots of both mixtures by titration with 0.004 *N* arsenious acid after addition of an excess of sodium bicarbonate. After 5 minutes 0.025 and after 90 minutes 0.0273 mm of periodate were consumed by the 0.0261 mm of diester, intermediate values being secured at the intervening times.

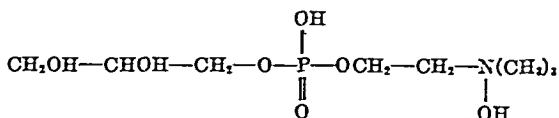
This behavior permits the conclusion that in the diester the glycerol group is esterified in the  $\alpha$  position.

*Optical Rotation*—A 2.57 per cent aqueous solution of the diester (calculated from its content of P) gave a rotation of  $-0.25^\circ$  in a 2 dm. tube.

$$[\alpha]_D^{25} = -4.87^\circ$$

*Action of Alkaline Phosphatase on  $\alpha$ -Glycerolphosphorylcholine*—0.5 cc. of a solution of  $\alpha$ -glycerolphosphorylcholine (containing 3.0 mg. of total P) was mixed with 10 cc. of 0.1 *N* veronal buffer of pH 9.3 and brought to a temperature of  $37^\circ$ . After the addition of 0.1 cc. of a solution of intestinal phosphatase (9) (activity, 0.002 cc. of phosphatase solution formed 0.9 mg. of P from glycerophosphate within 15 minutes under standard conditions) the mixture was incubated at  $37^\circ$ . The results are shown in Table IV.

The observations reported above suggest the following formula for the compound



which is analogous to that of the glycerolphosphorylcolamine obtained by Feulgen and Bersin (10) from acetal phosphatides, after treatment with mercuric chloride as catalyst.

*Yield*—Approximately 1 gm. of  $\alpha$ -glycerolphosphorylcholine is usually obtained from 5 pounds of beef pancreas. This yield accounts for only 10

to 15 per cent of the amount calculated from the data of Table I. The precipitation with ammonium reineckate and the treatment with Amberlite are mainly responsible for the losses during the isolation. In addition, the alcohol extraction of the incubated pancreas on a large scale does not yield more than approximately half as much of the soluble phosphorus fraction as the extraction of small amounts of pancreas with Bloor's mixture.

It is therefore possible that the crude alcohol-soluble petroleum ether-insoluble fraction of incubated pancreas contains other substances besides  $\alpha$ -glycerylphosphorylcholine. Furthermore, the fact that the isolated diester is the  $\alpha$  isomer does not exclude the possible presence of the  $\beta$  isomer in pancreas lecithin or among its enzymatic hydrolysis products.

TABLE IV  
*Action of Phosphatase on  $\alpha$ -Glycerylphosphorylcholine*

Time of incubation	Inorganic P formed	
min.	mg.	per cent total P of substrate solution
5	0.295	9.8
10	0.350	11.6
90	0.410	13.6

## DISCUSSION

The presence of  $\alpha$ -glycerylphosphorylcholine in incubated pancreas has a bearing on the interpretation of some earlier observations concerning the acid-soluble phosphorus compounds of this organ.

In 1939, King and Small (11) obtained from pancreas a phosphorus fraction which they considered as sphingosylphosphorylcholine. This assumption was based on the phosphorus and nitrogen content of their material and on the detection of choline among the products obtained by acid hydrolysis. They did not report any direct proof for the presence of sphingosine or the absence of glycerol in their material.

On the basis of our observations, we doubt whether the product analyzed by King and Small was a homogeneous substance. King and Small precipitated their material with ether from a protein-free pancreas filtrate after a series of barium, mercury, and lead salt precipitations. During all these procedures the material remained in the supernatants together with a large amount of contaminations. King and Small found that the composition of the final ether precipitate remained constant after several reprecipitations with acetone. We had a similar experience, but convinced ourselves that the precipitates obtained in this way were always mixtures. It was impossible to remove the nitrogenous contaminations from the

phosphorus compounds by reprecipitations with acetone. This could be accomplished only by the introduction of new steps of purification such as the precipitation of the phosphorus fraction by Reinecke acid and the adsorption of nitrogenous substances on Amberlite.

These considerations render it very unlikely that the material analyzed by King and Small was sphingosylphosphorylcholine. The analytical figures reported by these authors can be explained by the assumption that it was a mixture of glycerylphosphorylcholine and perhaps other phosphorus compounds with a nitrogenous material.

The connection of glycerylphosphorylcholine with the intermediary metabolism of lecithin is of considerable physiological interest.<sup>4</sup> It appears possible that this substance is important for the breakdown as well as for the synthesis of lecithin. In contrast to lecithin, which is not hydrolyzed by phosphatases, glycerylphosphorylcholine is split by alkaline phosphatase into its three constituents. It is possible that glycerylphosphorylcholine represents a water-soluble precursor of choline and choline derivatives in the mammalian organism.

The rate of this enzymatic hydrolysis is, however, very much slower than that of monoesters of phosphoric acid. For this reason, glycerylphosphorylcholine may accumulate in considerable amounts, even in organs with a high phosphatase content such as the small intestines. This fact, in connection with the very low glycerylphosphorylcholine content of the majority of the fresh tissues, suggests that glycerylphosphorylcholine may play an important part in the biological synthesis and in the "turnover" of lecithin in the intact organism.

Sinclair and Smith (12) discovered that ingested fatty acids are promptly incorporated into the phospholipids of the intestinal mucosa and the liver. The assumption that glycerylphosphorylcholine takes the part of a "fatty acid acceptor" in the biological synthesis of lecithin would furnish a simple explanation for the mechanism of the incorporation of fatty acids into the lecithin fraction of the digestive organs.

#### SUMMARY

1. The isolation of  $\alpha$ -glycerylphosphorylcholine from incubated beef pancreas has been described. Some of its physical and chemical properties have been investigated. It has been demonstrated that the isolated substance represents the  $\alpha$  isomer. The choline ester linkage of  $\alpha$ -glyceryl-

<sup>4</sup> The observation that the alcohol-soluble, petroleum ether-insoluble P fraction increases during the incubation not only in pancreas but also in some other organs, especially in intestinal mucosa, suggests the assumption that the formation of glycerylphosphorylcholine is not limited to the pancreas, but is of a more general importance for the metabolism of lecithin. It should be emphasized, however, that the isolation of glycerylphosphorylcholine has so far been carried out only on pancreas.

phosphorylcholine is very easily split by dilute acids. In this respect the behavior of glycerylphosphorylcholine differs sharply from phosphorylcholine, but resembles that of lecithin.  $\alpha$ -Glycerylphosphorylcholine is slowly hydrolyzed by alkaline phosphatase.

2. It has been shown that  $\alpha$ -glycerylphosphorylcholine originates from the enzymatic hydrolysis of lecithin. It is the only intermediary breakdown product of lecithin which has up to now been found in mammalian tissues.

3. Duodenal juice is without action on lecithin or cephalin, while intestinal mucosa is capable of hydrolyzing phospholipids at a rapid rate. The formation of  $\alpha$ -glycerylphosphorylcholine from lecithin is caused by the action of specific "lecitholipases" which are not identical with pancreas lipase.

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# THE STRUCTURAL SPECIFICITY OF SULFANILAMIDE-LIKE COMPOUNDS AS INHIBITORS OF THE *IN VITRO* CONVERSION OF INORGANIC IODIDE TO THYROXINE AND DIIODOTYROSINE BY THYROID TISSUE\*

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By means of radioactive iodine it was demonstrated in this laboratory that surviving slices of thyroid tissue can incorporate inorganic iodide into diiodotyrosine and thyroxine (1). When 300 mg. of thyroid slices were incubated for 2 hours in a Ringer's medium containing radioactive inorganic iodide, as much as 60 per cent of the labeled iodine was organically bound, about 50 per cent as diiodotyrosine and about 10 per cent as thyroxine. This *in vitro* reaction has provided a new and useful approach to the study of the biosynthesis of thyroxine. Thus, it was used to determine the mechanism by which the prolonged administration of the sulfonamides produces an enlargement of the thyroid gland, a hypertrophy that is characterized by hyperplasia and a loss of colloid. It was shown that at concentrations of  $10^{-3}$  M sulfanilamide, sulfapyridine, sulfathiazole, and sulfaguanidine depressed the synthesis of radiodiiodotyrosine and radiothyroxine (2). These compounds therefore act directly on the conversion of inorganic iodide to diiodotyrosine and thyroxine by thyroid tissue. *p*-Aminobenzoic acid and *p*-aminophenylacetic acid, because of their structural similarity to the sulfonamides, were also tested and found to be inhibitors (3).

In view of the importance of these findings to an understanding of the mechanism of biosynthesis of thyroxine, it became of interest to determine the structural part of the above molecules essential for their inhibitory action. Hence in the present investigation forty compounds structurally related to either sulfanilamide or *p*-aminobenzoic acid have been tested for their effects on *in vitro* formation of thyroxine and diiodotyrosine.

## EXPERIMENTAL

The procedures employed here have been described in earlier reports (1-3). Solutions of all compounds were prepared of such strength that by the addition of 0.1 cc. of these solutions to a 3.0 cc. volume of the bicarbonate-Ringer's solution a concentration of  $10^{-3}$  M was obtained. With

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the exception of tyrosine, all compounds were tested at a concentration of  $10^{-3}$  M. In the case of slightly soluble compounds it was found necessary to heat their solutions in order to obtain the desired concentrations, and in such cases 0.1 cc. of the hot solution was added to the radioactive Ringer's solution. The solutions of the compounds tested were adjusted to pH 7.0 to 7.5 before their addition to the buffered Ringer's medium.

### Results

*Aminobenzenesulfonic Acids and Amides and Compounds in Which Their Free Amino Group Has Been Replaced or Modified*—The extent to which *p*-, *m*-, and *o*-aminobenzenesulfonic acids and amides inhibited the conversion of radioactive inorganic iodide to thyroxine and diiodotyrosine by thyroid slices is shown in Table I. The para and meta isomers of the acid and amide were found to be quite active as inhibitors, the ortho compounds much less so.

No inhibitory effects were observed for either benzenesulfonamide or benzenesulfonic acid, compounds that contain no free aromatic amino groups.

Blocking of the primary amino group in sulfanilamide by an acetyl radical (*N*<sup>4</sup>-acetylsulfanilamide) resulted in considerable loss of activity. Replacement of the free amino group by a hydroxyl radical (*p*-hydroxybenzenesulfonamide) led also to a decrease in activity.

*Aminobenzoic Acids and Amides and Compounds in Which Their Free Amino Group Has Been Replaced or Modified*—The ortho, meta, and para isomers of aminobenzoic acid were found to be about equally active (Table I). Only a single amide was tested, namely *p*-aminobenzamide; it inhibited thyroxine and diiodotyrosine to the same degree as the above acids.

The removal of the amino group (benzoic acid) led to practically complete loss of inhibitory action.

The introduction of a hydroxyl group in the position para to that of the carboxyl radical in the benzoic acid molecule (*p*-hydroxybenzoic acid) conferred inhibitory activity, but the inhibition observed with this compound was less than that of the corresponding amino compound.

Blocking of the free amino groups of aminobenzoic acids by introduction of an acetyl radical resulted in considerable loss of activity. Acetyl-*p*-aminobenzoic acid showed practically no inhibition in the formation of thyroxine. Acetyl-*o*-aminobenzoic acid (*N*-acetylanthranilic acid) was found to be only weakly inhibitory.

*Compounds Related to Aniline and Phenol*—Aniline strongly inhibited the incorporation of inorganic iodide to diiodotyrosine and thyroxine. Little or no decrease in inhibitory activity was produced by substituting in the para position one of the following: a methyl group (*p*-toluidine),

an acetic acid side chain (*p*-aminophenylacetic acid), a hydroxyl group (*p*-aminophenol), or another amino group (*p*-phenylenediamine). The introduction of a nitro group (*p*-nitroaniline), however, did depress inhibitory activity considerably.

TABLE I

*Effect of Compounds Related to Sulfonilamide and p-Aminobenzoic Acid on Formation of Thyroxine and Diiodothyroxine*

All compounds were tested at a concentration of  $10^{-3}$  M except *l*-tyrosine for which the concentration was  $5 \times 10^{-4}$  M.

Compound	Per cent inhibition		Compound	Per cent inhibition	
	Thyroxine formation	Diiodothyroxine formation		Thyroxine formation	Diiodothyroxine formation
Sulfanilic acid	33	43	<i>p</i> -Toluidine	82	82
Sulfanilamide	47	58	<i>N</i> -Acetyl- <i>p</i> -toluidine	85	85
Metanilic acid	37	36	<i>N,N</i> -Dimethyl- <i>p</i> -toluidine	83	82
Metanilamide	84	85	<i>p</i> -Nitroaniline	20	0
Orthanilic acid	13	14	<i>p</i> -Aminophenylacetic acid	65	63
Orthanilamide	10	2	<i>p</i> -Phenylenediamine	74	74
Acetylsulfanilamide	18	10	<i>p</i> -Aminophenol	82	77
Benzenesulfonamide	0	0	<i>N</i> -Acetyl- <i>p</i> -aminophenol	57	51
<i>p</i> -Hydroxybenzenesulfonamide	10	16	<i>p</i> -Nitrophenol	86	84
Benzenesulfonic acid	0	0	Hydroquinone	80	79
Toluenesulfonic acid	6	19	" mono-methyl ether	80	76
<i>p</i> -Aminobenzoic acid	73	79	Hydroquinone dimethyl ether	27	9
<i>m</i> -Aminobenzoic acid	71	73	<i>p</i> -Hydroxyphenylglycine	80	75
<i>o</i> -Aminobenzoic acid (anthranilic acid)	63	63	Ethyl <i>p</i> -aminobenzoate (benzocaine)	82	87
<i>p</i> -Aminobenzamide	80	84	$\beta$ -Diethylaminoethyl <i>p</i> -aminobenzoate hydrochloride (procaine hydrochloride)	79	85
Benzoic acid	5	6	<i>l</i> -Tyrosine	9	0
<i>p</i> -Hydroxybenzoic acid	53	53	Taurine	0	0
<i>N</i> -Acetyl- <i>p</i> -aminobenzoic acid	0	9	<i>dl</i> -Phenylalanine	7	0
<i>N</i> -Acetyl- <i>o</i> -aminobenzoic acid ( <i>N</i> -acetyl anthranilic acid)	20	24	Sulfamic acid	0	0
Aniline	75	81			
Acetanilide	78	75			

As noted above, inhibition to the extent of 80 per cent was found in the case of *p*-aminophenol. Little or no effect on the inhibitory activity was produced when the amino group was replaced by either a nitro group (*p*-nitrophenol), a methoxy group (hydroquinone monomethyl ether),



a glycine side chain (*p*-hydroxyphenylglycine), or another hydroxy group (hydroquinone).

It was shown above that acetylation of the amino group of sulfanilamide and of *p*-aminobenzoic acid greatly reduced their inhibitory activities. This effect of acetylation is further borne out by comparing the inhibitory activities of *p*-aminophenol and acetyl-*p*-aminophenol. An intense inhibition (about 80 per cent) of diiodotyrosine and thyroxine formation by thyroid slices was observed in the presence of *p*-aminophenol and about 50 per cent in the presence of the same concentration of acetyl-*p*-aminophenol.

In contrast to the above findings it is of interest to note that acetylation of the amino group of aniline or of *p*-toluidine resulted in no depression of activity. Acetanilide and acetyl-*p*-toluidine inhibited diiodotyrosine and thyroxine formation to the extent of about 80 per cent.

Modification of the amino group of *p*-toluidine by the introduction of two methyl groups (dimethyl-*p*-toluidine) also failed to interfere with its inhibitory activity.

The results obtained with hydroquinone and its monomethyl ether on the one hand and hydroquinone dimethyl ether on the other hand provide a striking contrast in activity. Substitution of one of the hydroxyl groups of the hydroquinone molecule by a methoxy group led to no loss in inhibitory activity. When, however, both hydroxyl groups were replaced by methoxy groups, the resulting compound had but little inhibitory action.

*Esters of p-Aminobenzoic Acid*—These compounds are of interest because they are derivatives of *p*-aminobenzoic acid in which the carboxyl group has been esterified but the primary amino group has not been changed. The results show that modification of the carboxyl group does not affect inhibitory activity. Thus ethyl *p*-aminobenzoate (benzocaine) and  $\beta$ -diethylaminoethyl *p*-aminobenzoate hydrochloride (procaine hydrochloride) were found to be highly inhibitory.

The presence of  $5 \times 10^{-4}$  M *l*-tyrosine or of  $10^{-3}$  M *dl*-phenylalanine in the medium had little or no effect on the formation of radiiodotyrosine or radiothyroxine. When tested at a concentration of  $10^{-3}$  M, the aliphatic aminosulfonic acid, taurine, and the amide of sulfuric acid, sulfamic acid, also failed to show inhibitory action.

#### DISCUSSION

In 1943 Astwood tested 106 compounds for their goitrogenic activity (4). They were administered either with the food or with drinking water for 10 days, and the resulting degree of enlargement of the thyroid gland and proliferation of its acinar cells was used as a measure of inhibition

of thyroid function. It is interesting to compare the results obtained by this approach to thyroid activity with those provided by a procedure in which thyroid activity was determined by the rate at which surviving thyroid slices converted inorganic iodide to diiodotyrosine and thyroxine. Those compounds tested here which previously were judged by Astwood to be goitrogenic also inhibited the *in vitro* incorporation of inorganic iodide into diiodotyrosine and thyroxine. *But many compounds that failed to produce gross or histological changes in the thyroid gland when fed to the intact rat proved to be very active inhibitors of the in vitro formation of thyroxine and diiodotyrosine.* These included *p*-aminophenol, aniline, *p*-phenylenediamine, ethyl *p*-aminobenzoate, sulfanilic acid, and procaine. That these compounds, when ingested by the intact animal, are modified or changed before reaching the thyroid gland would appear to offer a satisfactory explanation for the discrepancy in the results obtained by the two methods.

Astwood postulated that compounds related to aniline are goitrogenic because of their structural similarity to tyrosine, and he suggested that aniline derivatives compete with diiodotyrosine for the enzyme systems responsible for its conversion to thyroxine (4). No support for this view is offered by the results of the present investigation. These compounds inhibited the synthesis of diiodotyrosine as well as that of thyroxine. If competition for the enzyme system that converts diiodotyrosine to thyroxine were the only mechanism involved, the observed degree of inhibition of the conversion of inorganic iodide to diiodotyrosine would not have occurred.

An examination of the results obtained here on the effects of closely related groups of aromatic compounds on the formation of thyroxine and diiodotyrosine permits of the following general conclusions concerning the requirements for inhibitory activity:

1. A free aromatic amino or free aromatic hydroxyl group favors inhibitory activity. Blocking of the amino group by acetylation reduces inhibitory properties in many instances. The presence of such radicals as the sulfonamide or the carboxyl confers no inhibitory properties.

2. The various isomers of aminobenzene sulfonic acid and of aminobenzenesulfonamide do not exhibit equal activities. The ortho isomers were least effective as inhibitors.

As a result of their investigations of the retardation of rancidity of fats by thiourea and other sulfhydryl compounds, György *et al.* (5) postulated that antioxygenic activity might explain the goitrogenic action of the sulfonamides and of *p*-aminobenzoic acid, as well as of thiourea and its derivatives. No experimental evidence, however, was offered in support of this view. This hypothesis is of interest here, for there appears to be

a correlation between the ease of oxidizability of compounds tested here and their capacity to stop the *in vitro* incorporation of inorganic iodide into diiodotyrosine and thyroxine. This is suggested by the following observations. (1) Many of the very active inhibitors contain either a free aromatic amino or hydroxyl group, structures that are conducive to oxidation. (2) Stabilization of the amino compounds either by acetylation or by elimination of the amino group reduces their inhibitory properties. (3) Hydroquinone and its monomethyl ether, both of which contain free phenolic groups and hence are easily oxidizable, are very active inhibitors. Hydroquinone dimethyl ether (in which both hydroxy groups are blocked, so that it is resistant to oxidation) had but little effect on the rate at which inorganic iodide is converted to thyroxine and diiodotyrosine.

The above considerations suggest that compounds shown to be inhibitory in the present study act by interfering with some oxidative step essential in the incorporation of inorganic iodide into diiodotyrosine and thyroxine. Since inorganic iodide must first be oxidized before it is organically bound (6, 7), it is possible that this is the oxidative step interfered with. If the incorporation of inorganic iodide into diiodotyrosine is a preliminary step in the formation of thyroxine, an interference in the oxidation of inorganic iodide would provide a satisfactory explanation for the interesting finding that all inhibitors interfered to about the same extent with both diiodotyrosine and thyroxine formation by thyroid slices.

The results obtained with several compounds do not support the view that inhibitory properties of a compound depend entirely on ease of oxidizability. Among these are acetanilide and acetyl-*p*-toluidine, both of which are quite resistant to oxidation. The inhibitory activities of these two compounds, however, might find an explanation in an enzymatic release of their amino groups by thyroid tissue.

These discrepancies, however, need not invalidate the general thesis expressed above. Differences in the rate of penetration of compounds into tissue slices may account for some of the discrepancies. Moreover, until such time as the reactions involved in the conversion of inorganic iodide to thyroxine are understood, there is need to guard against the assumption that all inhibitory compounds interfere with the same or even a single reaction.

#### SUMMARY

1. Forty compounds structurally related to the sulfonamides and aminobenzoic acid were tested for their effects on the *in vitro* conversion of radioactive inorganic iodide,  $I^{131}$ , to thyroxine and diiodotyrosine by surviving thyroid slices.

2. A free aromatic amino or hydroxyl group favored inhibitory activity. Blocking of the amino group by acetylation reduced inhibitory properties. The presence of such groups as the sulfonamide, the sulfonic acid, or the carboxyl was found to be unrelated to activity.

3. There appears to be a definite correlation between ease of oxidizability and inhibitory activity among the compounds tested.

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# THE REGENERATION OF LIVER PROTEIN IN THE RAT\*

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The liver weight in the rat rapidly decreases during short periods of fasting or feeding diets low in protein. Part of this loss in weight is due to the disappearance of liver protein, and the rate at which liver protein is lost is much greater than that of any other tissue thus far studied (1, 2). Early theories that the protein lost was a special storage or deposit protein have not been confirmed (3), and recent results indicate that the protein represents actual liver cytoplasm (4). The rapidity with which liver protein disappears during fasting and is restored with protein feeding (5) suggested that the rate of regeneration of this tissue protein might be used as a basis for the assay of the nutritional adequacy of proteins. Kosterlitz (4) found that the liver nitrogen in the rat, expressed in terms of body weight, was a function of the amount of protein fed as well as the type of protein. He offered the suggestion that, "The changes in cytoplasmic substance may also be determined, with a high degree of approximation, by estimating the non-glycogen non-lipin solids. This method may lend itself to a rapid assessment of the biological values of proteins."

In the present studies the effect of feeding various proteins on the regeneration of liver protein of the rat after fasting was determined. Proteins, the nutritional value of which had been tested by classical methods of assay, were chosen for these experiments.

Male rats of the Sprague-Dawley strain were used in these studies. They were kept on a stock synthetic diet for at least 1 week before the experimental period, and rats which had attained a body weight of 250 to 280 gm. were chosen. The composition of the diet is as follows: casein 20, corn-starch 42, Crisco 24, cod liver oil 2, yeast 8, and Osborne and Mendel salt mixture<sup>1</sup> 4 per cent, respectively. The rats were fasted 48 hours and then fed synthetic diets containing the protein to be tested. All of the experimental diets were isocaloric with the stock diet. In the instances in which fewer calories were furnished by protein, the carbohydrate was increased proportionately. 12 gm. of diet were given on the 1st day of re-feeding and 10 gm. on the subsequent 3 days. These amounts were found to be approximately the quantities which rats fed diets containing poor proteins would consume. The only variable among the diets was the type

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<sup>1</sup> Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 37, 572 (1919).

of protein used. Yeast protein, amounting to 3.9 per cent, was present in all of the diets.

At the end of the refeeding period, the animals were anesthetized with nembutal and exsanguinated from the abdominal aorta, in an attempt to obtain the livers with a minimum and relatively uniform residual amount of blood. The whole liver was removed, weighed in a drying dish, and dried to constant weight at 95°. The dried liver was then ground to a fine powder and aliquots taken for total nitrogen and total fat. Nitrogen was determined by the micro-Kjeldahl method. Total lipids were determined in the following manner. The dried powder was extracted for 1 hour with a 3:1 alcohol-ether mixture, filtered, the solvent evaporated off, and the

TABLE I  
*Rate of Regeneration of Liver Protein Following 48 Hour Fast*

Group	No. of rats	Body weight change during refeeding	Liver nitrogen	Liver nitrogen increment over fasting level
		gm.	mg. per 100 gm.	mg. per 100 gm.
Controls	13		120 $\pm$ 2.4*	
Fasted 48 hrs.	9		82 $\pm$ 1.6	
Refed 20% casein diet				
1 day	5	+12	96 $\pm$ 3.7	+14
2 days	5	+12	98 $\pm$ 2.0	+16
3 "	5	+16	105 $\pm$ 0.9	+23
4 "	7	+24	105 $\pm$ 1.8	+23
Refed 20% gelatin diet				
1 day	3	+12	89 $\pm$ 3.8	+7
2 days	3	+5	92 $\pm$ 1.5	+10
3 "	3	+5	80 $\pm$ 1.5	-2
4 "	4	-2	85 $\pm$ 3.5	+3

\* Mean  $\pm$  standard error of the mean.

residue reextracted with petroleum ether. The petroleum ether extract was filtered through a fat-free thimble, the petroleum ether removed by heating in a stream of air, and the petroleum ether-soluble material weighed.

A preliminary group of experiments was carried out to determine the optimal length of time for refeeding after the 48 hour fasting period. For this purpose both a protein known to be adequate in its amino acid composition, casein, and a grossly inadequate one, gelatin, were tried. The animals were fasted 48 hours and sacrificed 1, 2, 3, and 4 days after refeeding was begun. The results of these experiments are shown in Table I. Under the particular experimental conditions used in these studies, the loss in liver nitrogen after a 48 hour fast was approximately 32 per cent of

the original nitrogen content, confirming previous observations on the rapidity and extent of loss of liver protein. The control group consists of rats which had been on the stock synthetic diet for at least 1 week and sacrificed without any period of fast. All values are expressed in terms of mg. per 100 gm. of body weight. The reference weight used in the calculation for the fasted and refed animals is the body weight immediately before the fast.

The loss of body weight during the 48 hour fast was extremely constant in all groups of rats and was approximately 24 gm. After 4 days of feeding of the casein diet, the rats had regained their initial body weight, whereas the animals on the gelatin diet had begun to lose more weight. In the casein group, at the particular intake of nitrogen and calories chosen, the liver nitrogen had reached a plateau after 3 to 4 days of refeeding. After a similar period of feeding of the gelatin diet, the liver nitrogen value was approximately the same as in the fasted animals. 4 days of refeeding after the fast seemed to be an adequate period of time to reveal the difference between an adequate and a grossly inadequate protein in the effect on the rate of regeneration of liver protein. In succeeding experiments, therefore, various proteins were incorporated into the basic diet and fed for a standard period of 4 days following the 48 hour fast.

The effect of feeding diets containing various proteins on the regeneration of liver protein is shown in Table II. The nutritive value of the proteins tested has been studied by other methods by many investigators. Several of these proteins were fed at different levels in the diet. The amount of protein fed is given (Table II) as the total amount of protein per 100 gm. of body weight ingested by the animals during the entire refeeding period of 4 days. Casein and lactalbumin are seen to be identical with respect to their ability to promote liver protein regeneration. The feeding of diets containing 10 per cent of these proteins, supplying 1.5 gm. of protein per 100 gm. of body weight for the refeeding period, did not produce any significant increase in the liver protein above the amount found in the fasted rats. When the protein intake was doubled, the animals regained the body weight lost during the fast, and there was a considerable increase in liver nitrogen, but the values found were still below those of the controls. Only when diets containing 40 per cent of these proteins were fed for the 4 day period, supplying a total of 6.3 to 6.4 gm. of protein per 100 gm. of body weight, did the liver nitrogen increase to the prefasting levels. Body weight gain in this group was no greater than that in animals fed the 20 per cent protein diet.

In striking contrast to the results obtained with these adequate proteins are the findings in the rats fed gelatin. At all levels of protein intake there was practically no regeneration of liver protein. In the group fed the gela-



tin diet at the 40 per cent level, the total amount of 42 gm. of diet was not taken by some of the animals. The total protein and caloric intake was, therefore, somewhat lower in this group than in the comparable groups fed the casein and lactalbumin diets. The animals fed the gelatin diets did not regain any of the weight lost during the fasting period.

When diets containing 40 per cent of the vegetable proteins, zein and gliadin, were fed, the quantity of liver nitrogen at the end of the 4 day refeeding period was significantly less than that found when comparable

TABLE II  
*Influence of Amount and Type of Protein Fed on Liver Protein*

Group	No. of rats	Body weight change during refeeding	Liver nitrogen	Liver nitrogen increment over fasting level
		gm.	mg. per 100 gm.	mg. per 100 gm.
Controls	13		120 $\pm$ 2.4*	
Fasted	9		82 $\pm$ 1.6	
Casein diets				
1.5 gm. per period	9	+8	84 $\pm$ 1.6	+2
3.3 " " "	7	+24	105 $\pm$ 1.8	+23
6.3 " " "	6	+21	120 $\pm$ 4.0	+38
Lactalbumin diets				
1.5 gm. per period	5	+10	85 $\pm$ 3.8	+3
3.2 " " "	4	+23	102 $\pm$ 1.2	+20
6.4 " " "	6	+24	123 $\pm$ 1.9	+41
Gelatin diets				
1.6 gm. per period	4	+4	79 $\pm$ 1.6	-3
3.4 " " "	4	-2	85 $\pm$ 2.2	+1
5.1 " " "	6	+2	87 $\pm$ 1.5	+5
Zein diet, 6.4 gm. per period	7	+3	100 $\pm$ 2.8	+18
Gliadin diet, 6.4 gm. per period	6	+12	109 $\pm$ 1.4	+27
Whole egg protein diet, 3.3 gm. per period	6	+19	103 $\pm$ 1.5	+21
Liver diet, 3.3 gm. per period	6	+17	99 $\pm$ 1.3	+17

\* Mean  $\pm$  standard error of the mean.

amounts of casein or lactalbumin were given. Appreciable regeneration of liver protein occurred, in contrast to the lack of restoration of liver protein in the animals fed the gelatin diet at the same level of protein intake. The gain in body weight was less than that in the groups on the casein and lactalbumin diets. The quantity of liver nitrogen in the rats fed gliadin was slightly, but significantly, higher than in the group fed zein ( $p = 0.02$ ).<sup>2</sup> The gain in body weight was also significantly greater in the gliadin-fed rats than in the zein group ( $p < 0.01$ ).<sup>2</sup>

<sup>2</sup> Determined from Fisher's table of  $t$  for small samples (6).

In an attempt to determine whether rat liver protein would be more efficient in producing liver protein regeneration than casein or lactalbumin, dried rat livers from the various groups of animals were pooled and extracted with ether in a Soxhlet extractor. The fat-free residue was analyzed for nitrogen, and a diet was prepared containing 20 per cent of liver protein ( $N \times 6.25$ ). The amount of liver nitrogen found in rats fed this diet was approximately the same as that found in the casein and lactalbumin groups fed the same amount of protein.

A diet containing 20 per cent of whole egg protein was made from dried whole egg powder. The fat content of the egg powder was determined and

TABLE III  
*Effect of Feeding Low Protein Diets on Liver Protein*

Group	Rat No.	Food intake	Body weight change	Liver nitrogen
		gm.	gm.	mg. per 100 gm.
Controls				$120 \pm 2.4$
Fasted				$82 \pm 1.6$
Basal low protein diet	30			84
	31			79
	97	105	-3	97
	98	94	-1	86
	148	54	-11	71
	149	63	-21	80
3.5% whole egg protein diet	99	119	+20	108
	101	106	+24	96
	102	93	+3	90
	103	116	+24	94
3.5% egg white protein diet	100	110	+13	91
	104	150	+31	97
3.5% lactalbumin diet	150	67	-6	80
	151	50	-12	81
	152	86	+12	81

the amount of Crisco added was adjusted so that the total calories furnished by fat were the same as in the other diets. The increase in liver nitrogen in rats fed whole egg protein was the same as in the animals given casein or lactalbumin at a similar level.

Further experiments were carried out to determine the effect of very low protein intake on liver protein. In these studies there was no period of fast; the animals were taken off the stock synthetic diet, offered the experimental diet *ad libitum* for 1 week, and then sacrificed. The results of these studies are shown in Table III. Values for individual rats are given, since the food intake varied widely. The liver nitrogen is expressed as mg. per

100 gm. of the body weight at the end of the experimental period. The food intake represents the amount of diet consumed during the entire week. The basal low protein diet was one in which the sole source of protein was 8 per cent yeast powder, which on analysis was found to furnish 3.9 per cent yeast protein. The composition was as follows: corn-starch 62, Crisco 24, cod liver oil 2, yeast powder 8, and Osborne and Mendel salt mixture 4 per cent, respectively. Diets were made which contained 3.5 per cent whole egg protein, 3.5 per cent egg white protein, or 3.5 per cent lactalbumin substituted for an equivalent weight of corn-starch. These latter diets, therefore, contained 3.5 per cent of the indicated protein plus the 3.9 per cent yeast protein, and were isocaloric with the basal diet.

It may be seen that the liver nitrogen of rats fed for 1 week a diet containing as its sole source of protein 3.9 per cent yeast protein was decreased to values as low as those found in rats which had been fasted 48 hours. The amount of body weight lost is related to the food consumption. When a diet containing 3.5 per cent whole egg protein, in addition to the 3.9 per cent yeast protein, was fed for 1 week, there also occurred a marked loss in liver nitrogen. This loss in liver protein took place at a time when the animals were gaining weight. Similar results were obtained when egg white protein was fed at the same level. There was a marked loss of liver protein despite a substantial body weight increase. In the three rats fed the 3.5 per cent lactalbumin diet the food consumption was considerably lower than in the egg protein groups. The amount of liver nitrogen was decreased to the fasting level even in the one animal in which the food consumed was sufficient to permit gain in body weight.

Croft and Peters recently reported that the addition of 2 per cent methionine to a low protein diet greatly reduced the nitrogen loss of rats which had been subjected to thermal burns (7). It was, therefore, of interest to test the effect of methionine in similar quantities on the regeneration of liver protein, with the method of assay described. The amount of liver protein in rats fed a 10 per cent casein diet for 4 days following a 48 hour fast was no greater than that found in rats sacrificed immediately after the period of fasting. When a diet containing approximately the same nitrogen content as the 10 per cent casein diet, but with 2 per cent of the casein replaced by 2 per cent *DL*-methionine, was fed, a significant increase in liver protein above the fasting level was noted (Table IV). A control group of animals was fed a diet containing the non-essential amino acid, alanine, at a 1.2 per cent level (furnishing the same amount of  $\alpha$ -amino nitrogen as 2 per cent methionine). It is clear that mere substitution of an  $\alpha$ -amino acid for a corresponding amount of protein in this low protein diet does not result in an increase in the amount of liver protein. That the effect of methionine in increasing liver protein regeneration is not due to its rôle in

furnishing methyl groups may be seen from the results obtained when a diet containing 10 per cent casein and 0.5 per cent choline chloride (the latter offering the same concentration of methyl groups as 2 per cent methionine) was fed. The amount of liver protein found in these rats was the same as in the group fed the 10 per cent casein diet. Moreover, the addition of 2 per cent of *l*-cystine to the low casein diet resulted, as with methionine, in a significant increase in liver protein above that found in the 10 per cent casein group. Homocystine was prepared by Dr. J. Fishman, of this laboratory, according to the method of Butz and du Vigneaud (8), and 2 per cent of this amino acid was incorporated into the 8 per cent casein diet. Only three of the six rats fed this diet consumed the total amount of food offered, and the data on these animals are given. There

TABLE IV  
*Influence of Sulfur-Containing Amino Acids upon Liver Protein*

Group	No. of rats	Body weight change during refeeding	Liver nitrogen	Liver fat
		gm.	mg. per 100 gm.	gm. per 100 gm.
10% casein diet	9	+8	84 $\pm$ 1.6*	0.140 $\pm$ 0.003
8.8% " + 1.2% <i>dl</i> -alanine	6	+7	83 $\pm$ 2.3	0.144 $\pm$ 0.003
8% " + 2% <i>dl</i> -methionine	5	+12	100 $\pm$ 1.5	0.179 $\pm$ 0.018
8% " + 2% <i>l</i> -cystine	8	+15	95 $\pm$ 2.6	0.235 $\pm$ 0.010
8% " + 2% homocystine	3	+12	90	0.259
10% " + 0.5% choline chloride	6	+13	84 $\pm$ 1.5	0.142 $\pm$ 0.007
20% "	7	+24	105 $\pm$ 1.8	0.191 $\pm$ 0.003
18% " + 2% <i>dl</i> -methionine	5	+20	103 $\pm$ 3.1	0.178 $\pm$ 0.012

\* Mean  $\pm$  standard error of the mean.

appears to be some effect of this sulfur-containing amino acid on liver protein regeneration, but this series needs to be extended. It is interesting to note that the quantity of liver fat was increased in the rats fed the cystine and the homocystine diets. An increase in liver fat in rats, produced by the addition of cystine to low protein, high fat diets, has been described by Beeston and Channon (9) and Tucker and Eckstein (10). The sulfur-containing amino acids are not the only limiting factor in liver protein regeneration, as is shown by the results obtained when 2 per cent *dl*-methionine was substituted for 2 per cent casein in the 20 per cent casein diet. The increase in liver protein during the 4 day period of feeding was no greater than that found when the 20 per cent casein diet, without added methionine, was fed.

In the present studies the marked loss in liver weight associated with loss in liver protein during a 48 hour fast was noted, but it is evident that

TABLE V  
*Influence of Diet on Weight and Composition of Liver*  
 The results are given in gm. per 100 gm. of body weight.

Group	No. of rats	Liver weight	Water	Protein (N $\times$ 6.25)	Fat	Estimated glycogen
Controls	13	3.89 $\pm$ 0.074*	2.69 $\pm$ 0.051	0.745 $\pm$ 0.015	0.199 $\pm$ 0.009	0.20
Fasted 48 hrs.	9	2.38 $\pm$ 0.040	1.66 $\pm$ 0.030	0.514 $\pm$ 0.010	0.154 $\pm$ 0.008	
40% casein diet	6	3.58 $\pm$ 0.104	2.50 $\pm$ 0.074	0.744 $\pm$ 0.023	0.189 $\pm$ 0.011	0.05
40% lactalbumin diet	6	3.61 $\pm$ 0.089	2.52 $\pm$ 0.061	0.767 $\pm$ 0.012	0.192 $\pm$ 0.012	0.05
40% gelatin diet	6	2.86 $\pm$ 0.119	1.98 $\pm$ 0.084	0.540 $\pm$ 0.009	0.155 $\pm$ 0.009	0.13
Basal low protein diet	Rat No.					
	30	3.29	2.27	0.525	0.197	0.22
	31	3.36	2.37	0.494	0.166	0.25
	97	3.59	2.50	0.606	0.246	0.15
	98	3.52	2.43	0.537	0.192	0.28
	148	2.60	1.82	0.444	0.149	0.12
	149	3.33	2.28	0.500	0.256	0.21
	99	4.52	3.08	0.675	0.266	0.38
3.5% whole egg protein	101	3.68	2.53	0.600	0.178	0.27
	102	3.22	2.29	0.561	0.143	0.15
	103	3.50	2.44	0.586	0.230	0.16
3.5% egg white	100	3.64	2.49	0.569	0.218	0.28
"	104	3.82	2.60	0.605	0.239	0.28
3.5% lactalbumin	150	3.41	2.22	0.500	0.295	0.30
	151	2.84	1.91	0.506	0.286	0.06
	152	3.40	2.33	0.506	0.200	0.28

\* Mean  $\pm$  standard error of the mean.

the weight of the liver was only roughly related to the liver protein, expressed in terms of body weight. The major liver components were, therefore, determined: water, fat, protein, and glycogen which was estimated by difference. In the fasted rat in which the glycogen content was assumed to be essentially zero, the sum of the water, protein, and fat accounted for 97.5 per cent of the total liver weight. The sum of the salts and undetermined organic substances other than glycogen was, therefore,

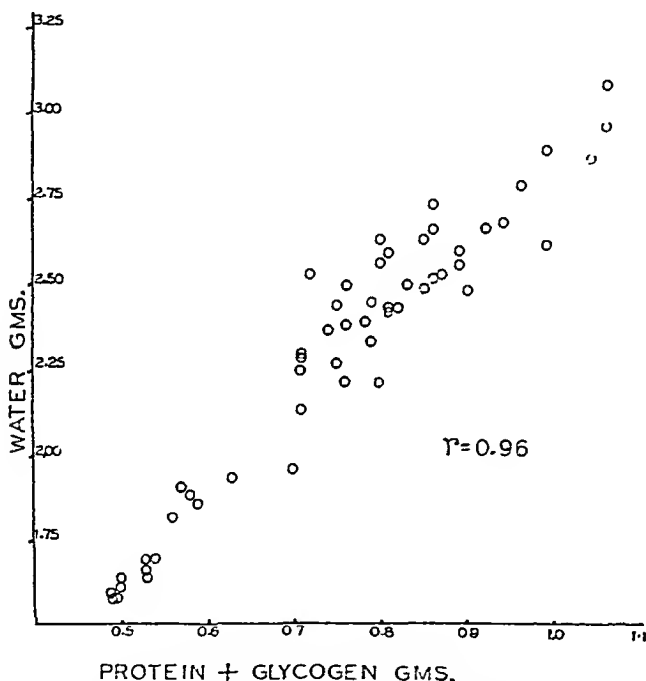


FIG. 1. The correlation between liver water and liver protein plus estimated glycogen.

assumed to represent 2.5 per cent of the liver weight. In the animals fed the various diets the glycogen could be roughly estimated by the following formula.

$$\text{Glycogen} = 0.975 \times \text{liver weight} - (\text{water} + (N \times 6.25) + \text{fat})$$

In Table V these components of the liver weight are tabulated, and all values are expressed in terms of gm. per 100 gm. of body weight. The

means of the groups are given for the control fed animals, the rats fasted for 48 hours, and the animals refed after a 48 hour fast, diets containing 40 per cent casein, lactalbumin, and gelatin respectively. The data for the individual rats which were fed low protein diets for 1 week without a preceding fast are also given. In these studies the differences in liver fat are relatively unimportant in proportion to changes in total liver weight. The fluctuations in the estimated liver glycogen, however, are considerable,

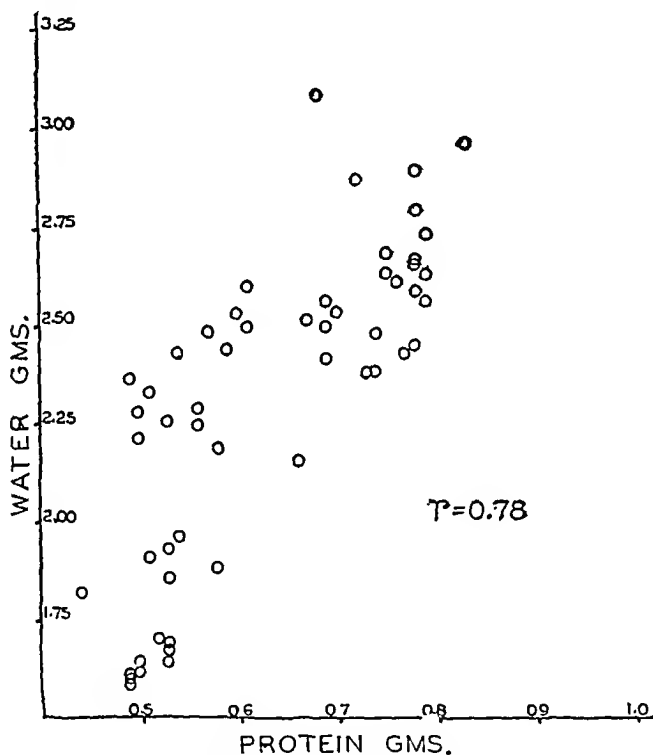


FIG. 2. The correlation between liver water and liver protein

and the loss of liver glycogen in the fasted animal is as important a factor in the reduction of liver mass as is the loss of liver protein. In rats refed 40 per cent casein or lactalbumin diets after a 48 hour fast, the liver protein returned to the control values, but the total liver weight was significantly lower than that of the control, and it may be seen that the estimated liver glycogen was low in these animals. In the animals fed a 40 per cent gelatin diet very little increase in the liver protein over the fasting level was observed, but there were some deposition of liver glycogen and increase in liver weight. In a number of the rats fed the low protein diet for 1 week,

the liver protein was reduced to levels as low as that found in the 48 hour fasted group, but the liver weight was considerably higher than that seen in the fasted animals because of the retention of liver glycogen. The influence of liver glycogen on liver weight is much greater than that due to the actual weight of glycogen in the liver, owing to the retention of water along with glycogen. This is shown by a comparison between total liver water and liver protein plus glycogen. The data for the individual animals of the groups listed in Table V are shown graphically in Figs. 1 and 2 with the corresponding correlation coefficients. There is a much better correlation between liver water and liver protein plus estimated liver glycogen than there is between liver protein alone and liver water. The linear nature of the former curve indicates that there was approximately the same amount of total liver water per gm. of liver glycogen present as per gm. of protein. These results are in agreement with the findings of Fenn on the relation of water and glycogen in the liver of rats (11).

#### DISCUSSION

From these studies it is evident that large quantities of an adequate protein must be fed before the liver protein, which has been lost during the 48 hour fast, is restored. When diets containing 20 per cent casein or lactalbumin were fed for a 4 day period following the fast, the amount of liver protein present was still below the control value, although the rats regained the body weight lost during the fast. This suggests that liver protein is not restored to its original level until all of the body protein lost is replaced. A combined study of liver protein and accurate nitrogen balances would be required to prove this point. The lability of liver protein is further illustrated by the findings in the animals fed the basal low protein diet supplemented by 3.5 per cent whole egg protein, egg white protein, or lactalbumin. Many of these rats showed a substantial gain in body weight during the time when liver protein was falling.

The data indicate that the regeneration of liver protein in the rat following a fast can be used as a method of assay of dietary protein. Rats of a uniform strain and within a fairly narrow weight range were used in these experiments. Under these conditions the total liver nitrogen, expressed in terms of body weight, is quite uniform, as is the loss of liver nitrogen during a 48 hour fast. The rate of regeneration of liver protein following this period of fast is clearly dependent upon the amount and the amino acid composition of the dietary proteins fed. If the amount of protein fed is kept constant, the differences between various proteins can be strikingly demonstrated. No regeneration of liver protein takes place in rats fed gelatin as the source of protein. Zein and gliadin, although incomplete proteins, are definitely superior to gelatin by this method of



assay. A statistically significant difference can be demonstrated between zein and gliadin, the latter being superior to zein. Both zein and gliadin are definitely inferior to casein and lactalbumin in promoting liver protein regeneration. These findings agree with the conclusions of other investigators using different methods of assay of food proteins (12).

In the present studies the amount of regeneration of liver protein obtained with casein and lactalbumin is identical. It has been shown, however, that in the rat larger amounts of casein than of lactalbumin must be fed to produce comparable growth (13). A significant difference between these proteins when tested by their influence on the regeneration of serum protein in the dog (14) has likewise been shown. The superiority of lactalbumin over casein as a food protein is a somewhat special case, since the deficiency of casein is due not to a lack of an essential amino acid but to a relatively low content of total sulfur-containing amino acids. In the growth experiments in rats the sulfur deficiency manifests itself only after a prolonged period of feeding. In the experiments involving serum protein regeneration a prolonged period of protein depletion was used before the test protein was fed. During these long experimental periods the lower sulfur content of casein as compared with lactalbumin could be demonstrated. In the present experiments rats fed an adequate diet were fasted for 48 hours prior to the refeeding for 4 days of the proteins studied. With such a short experimental period it is not surprising that no difference between casein and lactalbumin could be demonstrated.

The advantages of the determination of regeneration of liver protein as a method of assay of dietary protein are apparent. It is simple and rapid, and, with the precautions described, the uniformity of results is such that statistically significant differences can be demonstrated with small groups of animals. Further refinements of the technique are possible, particularly the substitution of crystalline components of the vitamin B complex for the yeast powder, thus eliminating yeast protein. The possible objection that the protein of a specific tissue, *i.e.* liver, might not be representative of the total body protein is not a real one, inasmuch as the results show that complete restoration of liver protein does not occur until the total body protein is replenished.

By the use of this method of assay, it has been possible to demonstrate that the addition of methionine or cystine to a diet containing an inadequate amount of a good protein, casein, will increase considerably the amount of liver protein regenerated after fasting. These results confirm the importance of methionine in protein synthesis demonstrated by Croft and Peters (7) by means of nitrogen balance studies in burned rats. Our results indicate further that the function of methionine in this process is not as a source of methyl groups, since the addition of choline to the same basal diet did not increase liver protein regeneration. It would appear that methionine

exerts its effect by virtue of its sulfur content, since cystine, and probably homocystine, acts similarly.

#### SUMMARY

1. A simple and rapid method for the assay of the nutritional adequacy of dietary protein is described, which is based on the regeneration of liver protein in the rat following a 48 hour fast.

2. Gelatin, zein, gliadin, casein, and lactalbumin were among the proteins assayed. The complete inadequacy of gelatin can be demonstrated by this method, as by the older procedures of assay. Zein and gliadin are superior to gelatin but can be shown by this technique to be incomplete proteins when compared with casein and lactalbumin. A significant difference can be demonstrated between zein and gliadin, the latter being superior to zein. No difference is found between casein and lactalbumin with respect to their ability to stimulate liver protein regeneration.

3. By the use of this method it has been shown that the addition of methionine or cystine to a diet containing an inadequate amount of casein will significantly increase the amount of liver protein regenerated after fasting.

*Addendum*—Since the preparation of this manuscript, a note has appeared by Kosterlitz and Campbell (15) which further confirms the validity of the use of liver protein regeneration as an index of amino acid composition of fed proteins. These authors have shown that in rats fed for 7 days a zein diet supplemented with both tryptophane and lysine, there was no loss of liver cytoplasm as measured by the non-lipid, non-glycogen solids. When either tryptophane or lysine was added singly to the zein diet, there was a decrease in liver cytoplasm, but not as great as when a protein-free diet was fed. The addition of tryptophane appeared to be more effective in sparing liver cytoplasm than lysine.

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## TRANSAMINASES

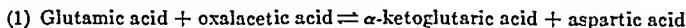
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Braunshtein and his coworkers (1) in their now classical investigations discovered the process of transamination in animal tissues and plants, and laid the foundation for an understanding of the enzymatic mechanisms by which this process takes place. They were led by their experiments to postulate the existence of two transaminating enzymes which they called glutamic and aspartic aminophosphatases, respectively. The glutamic enzyme reversibly transferred the amino group of *l*-glutamic acid to the keto acids of each of a considerable number of amino acids. Similarly, the aspartic enzyme was specific for *l*-aspartic acid as the primary donor of an amino group. Later, Cohen (2) took up the investigation of transamination, using a specific analytical method for the estimation of glutamic acid. While he confirmed the main features of the process of transamination as outlined by the Russian workers, he disagreed with them on the score both of enzyme specificity and the number of enzymes involved. Apart from alanine and glutamic and aspartic acids, Cohen found no other naturally occurring amino acid which underwent transamination at a significant rate. Furthermore, he was led to believe that glutamic and aspartic acids were dealt with by only one enzyme. He concluded that this enzyme was responsible for transamination between the pairs, glutamate-pyruvate and glutamate-oxalacetate.

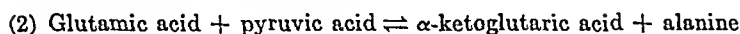
Much of the confusion and difficulty which have attended the study of transamination has been due either to inadequate analytical procedures or to procedures too laborious to permit the rapid testing of activity which is an essential preliminary to any successful enzyme study. In the present communication simple and rapid methods are described for the study of transamination. With the use of these methods we have been able to prosecute expeditiously the isolation of two transaminating enzymes from pig heart, each of which has been brought to a stage at which the bulk, if not all, of the protein present has transaminase activity. One of the enzymes catalyzes the reaction



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and will be referred to as the aspartic-glutamic transaminase. The other enzyme catalyzes the reaction



and will be referred to as the alanine-glutamic transaminase. Thus both transaminases share glutamic acid or its keto acid as an obligatory member of a transaminating pair. They differ only in their specificity for the second member of the pair. We have found no evidence for the existence of the glutamic acid and aspartic aminopherases with the specificities postulated by Braunshtein and Kritsman (1). Furthermore, neither of the two enzymes which we have isolated can catalyze transamination between the pair, aspartic acid-pyruvic acid. This reaction is only possible in the presence of the two enzymes and with glutamic or  $\alpha$ -ketoglutaric acid as catalyst. In other words, the reaction between aspartic acid and alanine is not direct but is rather a consequence of the two transaminating systems coming into equilibrium with one another.

### *Aspartic-Glutamic Transaminase*

*Assay of the Enzyme*—The action of the aspartic-glutamic enzyme may be followed by determining the formation or disappearance either of oxalacetic acid or of  $\alpha$ -ketoglutaric acid. The formation or disappearance of oxalacetic acid can be followed either manometrically or spectrophotometrically. Finally,  $\alpha$ -ketoglutaric acid can be readily oxidized to succinic acid by  $\text{H}_2\text{O}_2$  and then estimated manometrically by measuring the uptake of oxygen in the presence of the specific succinic oxidase.

Regardless of which method is selected for assay of the enzyme, the relation between the amount of chemical change and the amount of enzyme is not linear, except at the beginning of the reaction. The transamination reaction is reversible and its rate becomes progressively slower as the equilibrium point is approached. This difficulty can be by passed (a) by working with such an excess of substrate that a measurable amount of chemical change does not alter the initial concentrations appreciably, (b) by selecting for estimation an amount of enzyme which lies on that portion of the enzyme-concentration curve which is more or less linear, and (c) by carrying out the estimation of the enzyme over a relatively short period of time. By observing these precautions, the assay of transaminase can be made both accurate and consistent.

*Manometric Estimation of Oxalacetic Acid*—Ostern (3) has described a method of determining oxalacetic acid as  $\text{CO}_2$  which depends upon the decarboxylation of the keto acid in the presence of a concentrated aqueous solution of aniline adjusted to pH 4.6. If one prefers to work at  $38^\circ$ , the error arising from the decomposition of oxalacetic acid during the equi-

libration period can be eliminated by carrying out the transaminating reaction in which oxalacetic acid is formed, in the manometer cup, and then tipping the aniline reagent into the cup from a side arm. The  $\text{CO}_2$  evolved represents both the  $\text{CO}_2$  formed by decarboxylation of oxalacetic acid and the  $\text{CO}_2$  initially present in the reaction mixture as bicarbonate. The  $\text{CO}_2$  evolved from the control without transaminating enzyme should be equal to the bicarbonate  $\text{CO}_2$  initially present in the reaction mixtures. The control without the enzyme is preferred, since the enzyme solutions used are extremely dilute and the volume of enzyme solutions added is very small ( $< 0.1$  cc.). Thus, in practice no error is involved in neglecting the bicarbonate  $\text{CO}_2$  present in the enzyme solution.

The following mixture was set up in a Warburg manometer cup: 0.50 cc. of 0.2 M phosphate buffer of pH 7.3, 1.0 cc. of 0.2 M aspartate, 0.50 cc. of 0.2 M  $\alpha$ -ketoglutarate (in one of the side arms), and 0.1 cc. or less of enzyme solution. After temperature equilibration was attained, the taps were closed and the  $\alpha$ -ketoglutarate solution was tipped into the cup from the side arm. At the end of 10 minutes, 0.5 cc. of aniline citrate reagent<sup>1</sup> was tipped in from the second side arm or from the center well. Evolution of  $\text{CO}_2$  was complete within 10 minutes of the addition of the reagent.

Fig. 1 (Curve A) shows the relation between the amount of oxalacetic acid formed under the conditions mentioned above and the amount of enzyme. We have arbitrarily defined as a unit of transaminase activity an amount of enzyme which produces an amount of oxalacetic acid equivalent to 100 c.mm. of  $\text{CO}_2$  in 10 minutes at  $38^\circ$ . It appears that the best range for assay lies between 0.2 and 1.5 units. If the amount of oxalacetic acid formed is known, the amount of enzyme in transaminase units can be read directly from the curve.

For a given concentration of transaminase the amount of oxalacetic acid formed depends markedly upon the initial concentrations of aspartate and  $\alpha$ -ketoglutarate. Thus Curve B of Fig. 1 describes the relation when the concentrations of substrates were increased 2.5 times those obtaining for the standard curve, A. The importance of adhering rigorously to fixed initial concentrations of substrates becomes obvious.

*Spectrophotometric Estimation of Oxalacetic Acid*—The light absorption curves of  $\alpha$ -ketoglutaric, pyruvic, and oxalacetic acids are shown in Fig. 2. In the transformation of 1 micromole per cc. of  $\alpha$ -ketoglutaric acid to glutamic acid and of 1 micromole of aspartic acid to oxalacetic acid at pH 7.3, the density reading at  $280\text{m}\mu$  increases by 0.438 when the light path is 10 mm. Neither aspartic nor glutamic acid absorbs measurably at this

<sup>1</sup> The aniline citrate reagent was prepared by mixing equal parts of aniline and a solution of citric acid made up by dissolving 50 gm. of citric acid in 50 cc. of water.

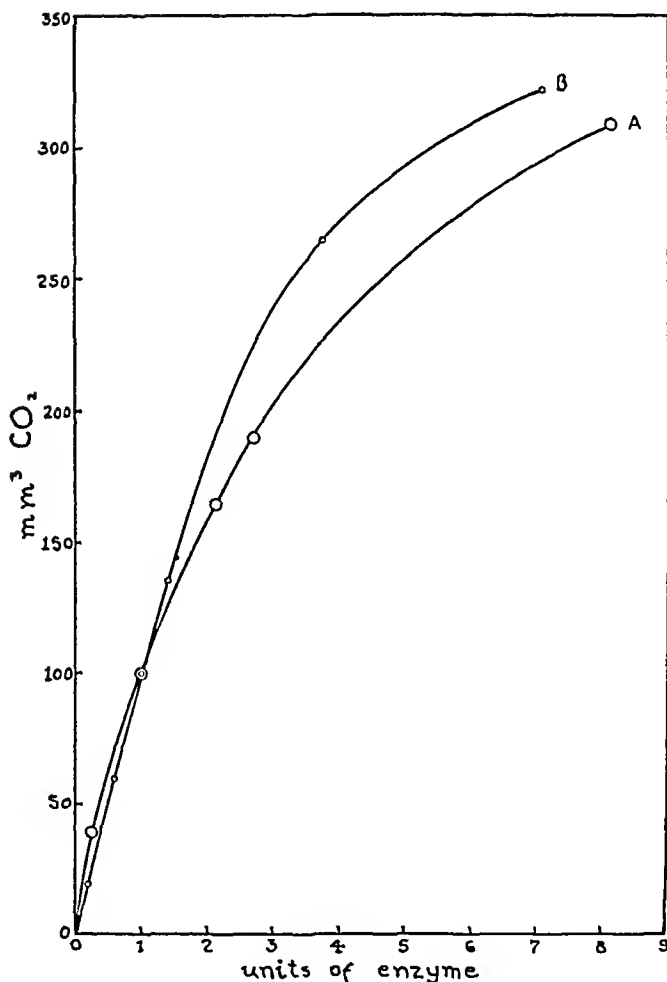


FIG. 1. Oxalacetic acid formation as a function of the concentration of aspartic-glutamic transaminase. The initial concentration of reactants in the experiments which make up the points of Curve A were 1.0 cc. of 0.2 M aspartate, 0.5 cc. of 0.2 M  $\alpha$ -ketoglutarate, and 0.5 cc. of 0.2 M phosphate buffer of pH 7.3. The experiment was begun after temperature equilibration in the bath at 38° by tipping in the aspartate solution from a side arm. After 10 minutes 0.4 cc. of aniline citrate reagent was tipped in from the center pot and the CO<sub>2</sub> evolution measured. In the experiments which make up the points of Curve B the initial concentrations of substrate were 2.5 M higher than in the corresponding experiments of Curve A.

wave-length. The value 0.438 represents the difference between the absorption of oxalacetic acid and that of  $\alpha$ -ketoglutaric acid. Fig. 3 shows the course of the reaction when oxalacetic acid either is formed or dis-

appears. The spectrophotometric method<sup>2</sup> makes it possible for the first time to follow the process of transamination almost continuously. The reaction under the conditions outlined in the legend of Fig. 3 is linear for about the first 10 minutes and then progressively decreases to zero as

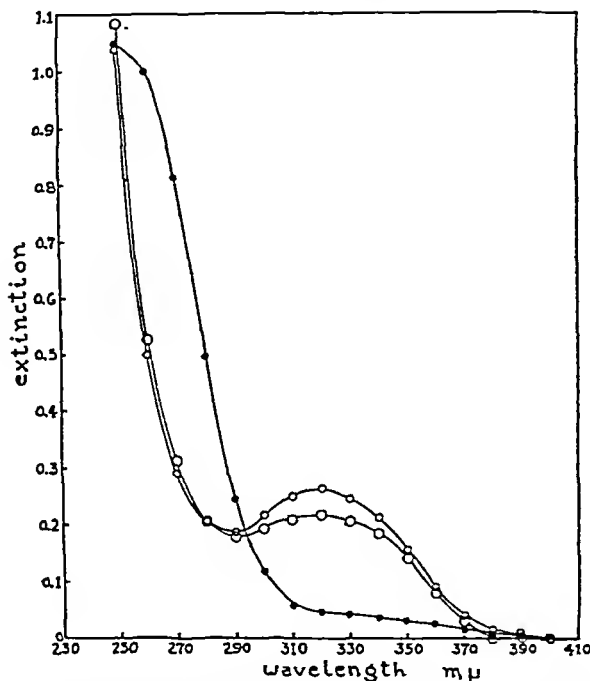


FIG. 2. Light absorption spectra of pyruvate, oxalacetate, and  $\alpha$ -ketoglutarate. The ordinates represent extinctions ( $\log I_0/I$  in a 1 cm. cell) at pH 7.3 and for the following concentrations of keto acid, oxalacetate (●) 1 micromole per cc.,  $\alpha$ -ketoglutarate (○) 10 micromoles per cc., pyruvate (O) 10 micromoles per cc.

the equilibrium point is approached. Oxalacetic acid decomposes spontaneously at an appreciable rate under the conditions of pH and tempera-

<sup>2</sup> The main drawback we have found to the more extensive use of the spectrophotometric method is the lack of provision for temperature control in the present model of the Beckman spectrophotometer. However, the National Technical Laboratories are in process of producing a unit for temperature control of the compartment housing the absorption cells. Given adequate temperature control, the spectrophotometric method would certainly be the method of choice for following the kinetics of aspartic-glutamic transaminase.



ture of the spectrophotometric test. It therefore becomes necessary either to correct for the spontaneous decomposition by running a control containing oxalacetic and all other components except the enzyme, or to insure the rapid attainment of the end-point within a few minutes after

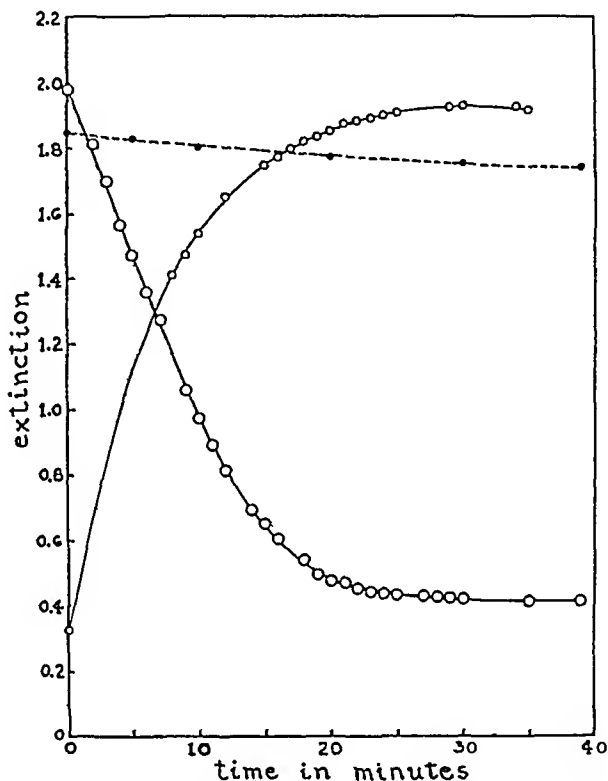


FIG. 3. The formation and disappearance of oxalacetate followed spectrophotometrically at  $280\text{ m}\mu$  in the glutamic-aspartic transaminase system. The curve O represents the formation of oxalacetate. The reaction mixture consisted of aspartate (10 micromoles per cc.), phosphate buffer of pH 7.3 (0.033 M), enzyme, and  $\alpha$ -ketoglutarate (10 micromoles per cc.) which was added at time zero. The length of the absorption cell was 1 cm. The curve O represents the disappearance of oxalacetate. The reaction mixture consisted of glutamate (8 micromoles per cc.), phosphate buffer of pH 7.3 (0.033 M), enzyme, and oxalacetate (3.7 micromoles per cc.) which was added at time zero. The curve ● represents the decomposition of oxalacetate (3.7 micromoles per cc.) at pH 7.3 in the presence of 0.033 M phosphate buffer.

the run has started by using larger amounts of enzyme. In the latter case the error due to the instability of oxalacetic acid can be neglected.

*Manometric Estimation of  $\alpha$ -Ketoglutaric Acid*—The sample of  $\alpha$ -ketoglutarate should not exceed 2 cc. and should not contain more than 30

micromoles. The sample under test (previously boiled to inactivate any catalase, if this is present) is introduced into a test-tube and 0.2 cc. of 3 per cent  $H_2O_2$  is added. The oxidation is allowed to proceed for 10 minutes at room temperature. 1 drop of paraffin oil is added (the use of capryl alcohol is to be avoided because it inhibits the succinic enzyme), and then a drop of a concentrated solution of catalase. When the foaming has stopped, the tube is heated in a boiling water bath for 3 minutes. Then a 2 cc. aliquot is transferred to a Warburg manometer cup containing 0.2 cc. of 6 N sodium hydroxide in the center well, 0.5 cc. of a concentrated preparation of succinic dehydrogenase in the side arm, and 0.5 cc. of phosphate buffer of pH 7.3 in the main compartment of the cup. After temperature equilibration, the succinic enzyme is tipped into the main compartment and the oxygen uptake is recorded when no further change ensues (usually 1 to 2 hours). 1 atom of oxygen is equivalent to 1 molecule

TABLE I  
*Estimation of  $\alpha$ -Ketoglutaric Acid*

$\alpha$ -Ketoglutaric acid added	Oxygen uptake, observed	Oxygen uptake, theory
<i>micromoles</i>	<i>c.mm.</i>	<i>c.mm.</i>
10	104	112
13.3	150	149
16	186	179
20	235	224
20	228	224
26.5	286	297
30	336	336

of  $\alpha$ -ketoglutaric acid. Table I shows the results of some typical estimations of known samples of  $\alpha$ -ketoglutaric acid by the above manometric method.

*Preparation of Reagents*—The following preparations were used: succinic enzyme (4), catalase (5, 6), lithium pyruvate (7),  $\alpha$ -ketoglutaric acid (8), and oxalacetic acid (9).

*Preparation of Enzyme*—Pig heart is the richest and most convenient source of glutamic-aspartic enzyme that we have found. The enzyme is stable indefinitely when the fresh hearts are kept frozen on dry ice and also in solution at all stages of purification. We have used as our index of purity the ratio of light extinction at 280  $m\mu$  in a 1 cm. cell to the number of enzyme units per cc. (10). The conditions for measuring enzyme units are given in the legend for Curve A, Fig. 1.

*Preparation of Aspartic-Glutamic Transaminase*—Pig hearts (10.2 kilos) were minced finely and mixed with 4 volumes of water. The suspen-

sion was stirred for 30 minutes and then filtered through several layers of fine gauze. The residue was pressed out and washed with 2 liters of water. The combined filtrates (42.4 liters) contained 14.3 units per cc., purity index 0.42, total number of units 608,000. Ammonium sulfate (30 gm. per 100 cc.) was added and the precipitate filtered and kept for the preparation of the alanine-glutamic enzyme. The filtrate was treated again with ammonium sulfate (40 gm. per 100 cc.) and the precipitate was filtered through fluted papers. The papers were stirred in 3 liters of water and the mixture was filtered through layers of fine gauze. The residue was pressed out and washed several times with 0.4 liter of water. The combined filtrates had a volume of 5 liters, of which 1.6 liters represented the volume of the ammonium sulfate precipitate. The neutralized solution contained 18.8 units per cc.,<sup>3</sup> purity index 0.85, total number of units 94,000.

TABLE II  
*Determination of Optimum Amount of Gel to Be Added*

Volume of gel added per volume of enzyme solution	Enzyme units* per cc. corrected for dilution with gel	Purity index*
0	70	0.25
0.5	70	0.097
1.0	56	0.086
1.5	52	0.081
2.0	48	0.096

\* As defined in the text.

Ammonium sulfate (1.2 kilos) was stirred slowly into the enzyme solution to make the final concentration 46 gm. per 100 cc. The precipitate was filtered off through fluted papers and dissolved by the addition of 500 cc. of water. The solution (640 cc.) containing 105 units per cc., purity index 0.45, total number of units 67,000, was heated to 60° in a boiling water bath and then filtered through fluted papers. The filtration is usually rather slow, requiring at least 12 hours for completion. The filtrate (500 cc.) contained 90 units per cc., purity index 0.25, total number of units 45,000. The enzyme solution was then dialyzed for 6 hours against running tap water in a Visking sausage casing, final volume 620 cc., 72 units per cc., purity index 0.25.

<sup>3</sup> In testing dilute solutions of the enzyme containing considerable amounts of ammonium sulfate, the estimation may be in error owing to the inhibition of the enzyme activity by ammonium sulfate. This inhibition becomes apparent when the estimated number of units per cc. increases as the volume of the sample used for the test becomes smaller. Dialysis for 5 hours against running water suffices to reduce the level of ammonium sulfate below the inhibitory level.

The dialyzed solution was then treated with tricalcium phosphate gel (11). A trial run to determine the optimum amount of gel to be added was carried out as indicated in Table II. Hence the enzyme solution was mixed with 1.5 volumes of tricalcium phosphate gel. The supernatant fluid after removal of the gel by centrifugation was treated with ammonium sulfate (70 gm. per 100 cc.). The precipitate was filtered through fluted papers and dissolved by addition of 100 cc. of water. The concentrated solution of the enzyme was then fractionated by stepwise addition of ammonium sulfate into five fractions (Table III). Fraction IVa, con-

TABLE III  
*Fractionation of Concentrated Enzyme Solution*

Fraction No.	Volume	Units per cc.	Purity index
	cc.		
Ia	11.4	5	4.38
IIa	12	15	1.18
IIIa	25	94	0.25
IVa	24	650	0.048
Va	20.8	77	0.143

TABLE IV  
*Refractionation of Fraction IVa*

Fraction No.	Volume	Units per cc.	Purity index
	cc.		
Ib	10	32	0.265
IIb	10	156	0.135
IIIb	10	89.5	0.108
IVb	10	1060	0.043
Vb	10	36	0.153

taining 15,600 units with a purity index of 0.048, was again fractionated into five fractions with ammonium sulfate (Table IV).

Fraction IVb, containing 10,600 units of purity index 0.043, was found in the Tiselius apparatus at pH 7.4 to contain three components with the following mobilities: 0.94, 2.87, and  $4.90 \times 10^{-5}$  sq. cm. gm.<sup>-1</sup> sec.<sup>-1</sup>. These were separated, and activity was found to be associated with both the 4.90 and 2.87 components of Fraction IVb which, respectively, accounted for 45 and 35 per cent of the total protein. The 0.94 component was found to be inactive. It should be pointed out that by using another and less satisfactory method of purification a different electrophoretic picture was obtained at the stage of purity index 0.043. In this case the ratio of the two components with velocities of 2.87 and 4.90 respec-

tively was 7:1 instead of 1.3:1 as above. Apparently the relative proportions of these two active components are determined by the method of purification. After electrophoretic separation of the 0.94 component the preparation of the enzyme has a purity index of 0.037, and appears homogeneous in the ultracentrifuge. The fact that the aspartic enzyme occurs in two forms of equal catalytic activity is not unique. The *l*-amino acid oxidase has recently been shown to occur in the form either of a unit with a molecular weight of 120,000 or one of 480,000; these forms have identical catalytic properties (12).

The first aqueous extract in the above preparation was found to contain 608,000 units. Only 94,000 units were recovered after the solution was fully saturated with ammonium sulfate. The first precipitate (30 gm. per 100 cc.) contained only insignificant amounts of the enzyme. There is always some loss associated with the precipitation of an enzyme from a dilute solution by full saturation with ammonium sulfate, but in this instance 85 per cent of the initial activity is lost. We are inclined to the interpretation that the estimate of the amount of the enzyme in the first extract is fictitiously high. The basis for this interpretation is the change in the purity index before (0.42) and after (0.85) precipitation by full saturation with ammonium sulfate. One would expect the precipitated enzyme to be purer rather than less than half as pure as it was in the original extract. This discrepancy has yet to be explained.

The initial amount of enzyme per 2 kilos of dry weight of pig heart muscle is about 750,000 units, allowing for the volume of the insoluble residue after making the first aqueous extract of the minced muscle. This amount of aspartic transaminase corresponds to 31 gm. or 1.6 per cent of the dry weight of muscle. If the estimate of the number of enzyme units in the first extract is too high, as suggested above, the estimate of the concentration of transaminase in the intact muscle would have to be lowered proportionately.

*Constants of Aspartic Transaminase*—At the stage of purity index 0.037, 1 mg. of enzyme is equivalent to 24 units. Since 1 unit represents an amount of oxalacetic acid equivalent to 100 c.mm. of  $\text{CO}_2$  formed per 10 minutes at  $38^\circ$ , the  $Q_{\text{co}_2}$  (the  $\text{CO}_2$  equivalent formed per hour per mg. of dry weight of enzyme) is 14,400. On the basis of a molecular weight of 60,000 for glutamic-aspartic transaminase, calculated from the diffusion and ultracentrifuge data of Dr. D. Moore, each molecule of enzyme would catalyze about 640 transamination reactions per minute. Finally, the enzyme at the stage of purity index 0.037 in a concentration of 1 mg. per cc. in a 1 cm. cell has an extinction of 1.07 at 280  $\text{m}\mu$ .

*Specificity of Enzyme*—The glutamic-aspartic transaminase is highly specific for the two naturally occurring dicarboxylic amino acids. No

other amino acid has been found to be active as a donor of amino groups to  $\alpha$ -ketoglutaric acid. Alanine, leucine, serine, and methionine cannot replace aspartic acid. Glutamine and pyrrolidinecarboxylic acid cannot replace glutamic acid. Mesoxalic acid can replace oxalacetic acid, but the rate of transamination in the presence of mesoxalic acid is only a small fraction of that in the presence of oxalacetic acid. Similarly cysteine acid can replace aspartic acid, though again very feebly.

#### *Alanine-Glutamic Transaminase*

*Assay of Enzyme*—The activity of the alanine-glutamic enzyme may be followed by determining the formation or disappearance either of  $\alpha$ -ketoglutaric acid or of pyruvic acid. The manometric estimation of  $\alpha$ -ketoglutaric acid has been described above.

*Colorimetric Estimation of Pyruvic Acid*—Straub's method (13) consists of condensing pyruvic acid with salicylaldehyde in strongly alkaline solution to form a colored substance with a light absorption peak at 440  $m\mu$  in the visible spectrum. Neither  $\alpha$ -ketoglutaric acid nor oxalacetic acid reacts with the reagent under these conditions. 1 cc. of the pyruvic acid solution containing not more than 5 micromoles was mixed in order with 1.0 cc. of KOH solution (100 gm. plus 60 cc. of water) and exactly 0.5 cc. of salicylaldehyde solution (2 per cent in alcohol). After 10 minutes incubation at 38°, the solution was made up to 25 cc. with water and the extinction at 440  $m\mu$  was determined against a blank with all additions except pyruvic acid. Fig. 4 shows that a linear relation obtains for the range of concentrations investigated.

For estimation of the activity of the enzyme a reaction mixture was set up consisting of 0.5 cc. of 0.2 M phosphate buffer of pH 7.3, 0.2 cc. of 0.2 M  $\alpha$ -ketoglutarate, and enzyme solution in a volume not exceeding 0.2 cc. The mixture was warmed in a water bath at 38° for 5 minutes. Then 0.2 cc. of M *dl*-alanine solution (also kept at 38°) was introduced and the transamination reaction allowed to proceed for 10 minutes, at which time the KOH solution was added. From then on the procedure is identical with that described for the estimation of pyruvic acid. A blank with all additions except that of enzyme was included and the extinction of the experimental at 440  $m\mu$  was measured against this reagent blank. Since the color of the salicylaldehyde-pyruvic acid condensation product is not stable, depending on factors which are not easily reproduced from one set of estimations to another, a pyruvic acid standard was run through the estimation procedure simultaneously with the experimentals. Fig. 5 shows the relation between the amount of enzyme and the amount of pyruvic acid formed. An approximately linear relation obtains when the enzyme is added in an amount which does not produce more than 5

micromoles of pyruvic acid in 10 minutes under the conditions of the test. We have arbitrarily defined as a unit of glutamic-alanine activity an amount of enzyme which forms 4.47 micromoles of pyruvic acid per 10 minutes. This apparently odd quantity is equivalent to 100 c.mm. of  $\text{CO}_2$  and thus the unit of activity is identical with that of the glutamic-aspartic enzyme.

*Preparation of Enzyme*—The unit of activity and the index of purity for the glutamic-alanine enzyme are defined in the same way as for the

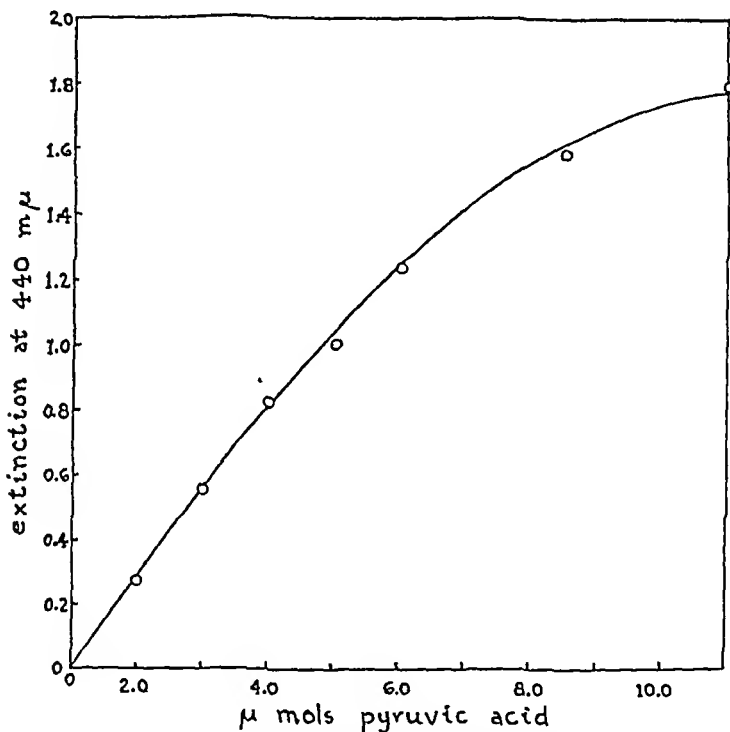


FIG. 4. Relationship between the light absorption at 440  $m\mu$  and the amount of pyruvic acid. Increasing amounts of lithium pyruvate were mixed with salicylaldehyde in alkaline solution, as described in the text. The density readings were made in a 1 cm. cuvette at 440  $m\mu$ .

other transaminase. The glutamic-alanine enzyme is comparatively less stable in pig heart and occurs in lower concentrations. The precipitate formed by addition of 30 gm. of ammonium sulfate per 100 cc. in the first step in the purification of the glutamic-aspartic enzyme serves as the starting point of the preparation.

*Preparation of Alanine-Glutamic Transaminase*—Fresh or frozen pig hearts (6.8 kilos) were minced finely and mixed with 4 volumes of water.

After being stirred for 30 minutes, the suspension was filtered through layers of fine gauze. The filtrate (27 liters) contained 5 units per cc., purity index 0.94, total number of units 137,000. Ammonium sulfate was added (30 gm. per 100 cc.) and the precipitate was filtered off through fluted papers. The papers were stirred in 1 liter of water and the mixture was filtered through layers of fine gauze. The residue of filter paper was pressed out and washed several times with water until the washings were

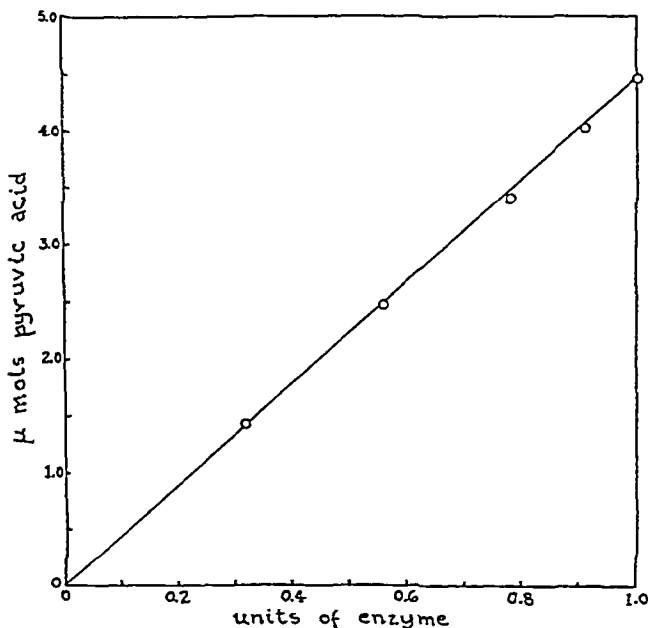


FIG. 5. Pyruvic acid formation as a function of the concentration of alanine-glutamic transaminase. The initial concentrations of reactants were 0.2 cc. of 0.2  $M$   $\alpha$ -ketoglutarate, 0.2 cc. of  $M$  alanine, and 0.5 cc. of 0.2  $M$  phosphate buffer of pH 7.3. Total volume 1 cc., reaction time 10 minutes, 35°.

colorless. The filtrate (1.11 liters) contained 72 units per cc., purity index 0.32, total number of units 80,000.<sup>4</sup>

The above filtrate was neutralized to pH 7 with 6  $N$  sodium hydroxide and rapidly heated to 60° in a bath of boiling water. The coagulum was removed by filtration or centrifugation and washed thoroughly with water. The turbid filtrate or supernatant was mixed with ammonium sulfate

<sup>4</sup> Cf. foot-note 3.



(30 gm. per 100 cc.). The precipitate was centrifuged down and redissolved by addition of 200 cc. of water. The enzyme solution is now sufficiently concentrated to permit of fractionation by stepwise addition of ammonium sulfate. The solid reagent is added slowly with vigorous stirring until a precipitate is formed. The precipitate is centrifuged down and the supernatant fluid is again treated with ammonium sulfate. In this manner three fractions were obtained, only the first of which (Fraction Ia) contained any appreciable amount of activity, volume 116 cc., 390 units per cc., purity index 0.22, total number of units 45,000.

The solution of Fraction Ia was dialyzed for 4 hours against running tap water and then for 12 hours at 1° against distilled water. The bulky

TABLE V  
*Refractionation of Fraction Ia*

Fraction No.	Volume	Units per cc.	Purity index	Total No. of units
	cc.			
Ib	40	296	0.101	11,800
I Ib	18	480	0.087	8,600

TABLE VI  
*Refractionation of Fraction Ib*

Fraction No.	Volume	Units per cc.	Purity index	Total No. of units
	cc.			
Ib <sub>1</sub>	17	290	0.153	4900
Ib <sub>2</sub>	17	270	0.071	4600
Ib <sub>3</sub>	17	88	0.107	1500

precipitate which formed on dialysis was centrifuged off and washed. The combined supernatant fluids (168 cc.) contained 208 units per cc. of purity index 0.098, total number of units 35,000.

Stepwise fractionation with ammonium sulfate was now repeated. Four fractions were obtained, Fractions Ib, I Ib, IIIb, and IVb, only two of which were active, *viz.*, Fractions Ib and I Ib (Table V). Fraction Ib was refractionated into three fractions with the results indicated in Table VI. Fractions Ib<sub>2</sub> and I Ib were combined and refractionated into six fractions with ammonium sulfate (Table VII). Fractions IIc and IIIc were mixed and refractionated with ammonium sulfate into five fractions (Table VIII). Fractions I Id and IVd were separately fractionated into three fractions each. The last two fractions of I Id and the first fraction of IVd were combined with Fraction IIIb, and the resulting solution was

fractionated with ammonium sulfate for the fifth and final time into three fractions (Table IX). We have been unable to advance the purity of the enzyme beyond the stage of purity index 0.020 by chemical fractionation procedures. While the preparation of the enzyme at this stage appears homogeneous in the ultracentrifuge, two components can be seen

TABLE VII  
*Combination and Refractionation of Fractions Ib<sub>2</sub> and IIb*

Fraction No.	Volume	Units per cc.	Purity index	Total No. of units
	cc.			
Ic	6	372	0.052	2200
IIc	12	670	0.029	8000
IIIc	11	576	0.039	6400
IVc	11	85	0.195	940
Vc	11	0	∞	0
VIc	11	0	∞	0

TABLE VIII  
*Refractionation of Combined Fractions IIc and IIIc*

Fraction No.	Volume	Units per cc.	Purity index	Total No. of units
	cc.			
Id	4	216	0.067	865
IIId	4	712	0.0316	2850
IIIId	4	950	0.0213	3800
IVd	4	684	0.0344	2740
Vd	4	70	0.25	280

TABLE IX  
*Fractionation of Combined Fractions of Fractions IIId and IVd*

Fraction No.	Volume	Units per cc.	Purity index	Total No. of units
	cc.			
Ie	3	567	0.020	1700
IIe	5	950	0.020	4900
IIIe	4.5	194	0.045	875

in the Tiselius apparatus. An appreciable amount of the smaller and faster moving component can be separated after 12 to 24 hours from the other component. The purity index of this electrophoretically homogeneous component was found to be 0.008. The second component was inactive.

The initial yield of enzyme from 1.36 kilos of dry weight of minced heart muscle is 171,000 units. Since 107 units are equivalent to 1 mg.,

the amount of alanine enzyme present initially is 1.6 gm. or about 0.12 per cent of the dry weight of heart muscle.

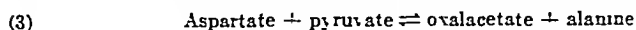
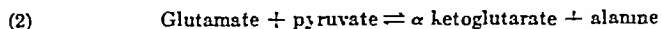
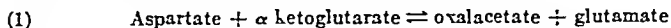
*Constants of Alanine-Glutamic Enzyme*—At the stage of purity index 0.008, 1 mg. of enzyme is equivalent to 107 units ( $Q_{co}$ , of 64,000). The activity per unit weight of the glutamic-alanine enzyme is therefore about 4.5 times that of the glutamic-aspartic enzyme under comparable conditions. Assuming a molecular weight of 180,000, each molecule of the glutamic-alanine enzyme would catalyze 8600 transamination reactions per minute at 38° under the conditions of the standard test. This turnover number is some 13 times greater than that of the glutamic-aspartic enzyme. 1 mg. of homogeneous enzyme per cc. in a 1 cm. cell has an extinction at 280  $m\mu$  of 0.86.

*Specificity of Enzyme*—In the reaction between alanine and  $\alpha$ -ketoglutaric acid, alanine can be replaced by  $\alpha$ -aminobutyric acid, though the velocity is only a small fraction of that with alanine. N-Monomethylalanine, phenylalanine, valine, serine, methionine, leucine,  $\alpha$ -aminovaleric acid, cysteic acid, and *d*-alanine cannot replace *l*-alanine. In the reaction between glutamic acid and pyruvic acid the latter can be replaced by  $\alpha$ -ketobutyric acid and mesoxalic acid, while the former cannot be replaced by cysteic acid, glycyl cysteine, glutathione, pyrrolidonecarboxylic acid, acetylglutamic acid, leucine, methionine, glutamine, tyrosine, threonine,  $\alpha$ -aminocaproic acid, lysine, phenylalanine, cystine, valine, and hydroxyproline.

*Equilibrium Constants*—In the reactions between  $\alpha$ -ketoglutarate and aspartate or alanine, an equilibrium is established, as was clearly shown by Kritsman (14) and Cohen (15). Using enzymes of the highest purity level, we have confirmed the approximate positions of the equilibria which Cohen reported for the two transaminating reactions,  $K = 0.3$  for the reaction shown below in reaction (1), and  $K = 1$  for the reaction shown in reaction (2). In the calculation of the equilibrium constant, the error in estimating any one of the four reactants is squared. Since the errors of estimation lie within the range of 5 to 10 per cent the uncertainty in evaluating the equilibrium constant is 25 to 100 per cent. With the present methods of estimation there is little point in assigning other than tentative values for the equilibrium constants of the two transaminating reactions.

*Prosthetic Group of Transaminases*—Braunshtein and Kritsman (16) have reported that the so called aspartic aminopherase contains a dissociable coenzyme, and they have reported on the preparation of this coenzyme from yeast. We have been unable to find any evidence that a transaminase exists in animal tissues which is specific for aspartic acid and not for glutamic acid. It is our impression that the so called aspartic enzyme is merely a mixture of the two transaminases with glutamic or  $\alpha$ -keto-

glutaric acid as "catalyst." Thus reaction (3) between aspartate and pyruvate is the summation of reactions (1) and (2):



The mode of preparation and the properties of the coenzyme which Braunschtein and Kritsman have isolated from yeast strongly suggest that it is glutamic acid. In other words, the coenzyme effect can be explained entirely in terms of reactions (1) and (2).

Although no evidence has been found that either of the two transaminases becomes inactivated during the purification procedure as the result of the loss of a dissociable coenzyme, we have been able to show that both enzymes contain a substance, some of whose properties are known to be shared by a derivative of pyridoxal. But first it will be necessary to digress to a consideration of the properties of dihydroxyphenylalanine or dopa carboxylase<sup>5</sup> of pig kidney. This carboxylase on purification was found to be inactive unless supplemented with a coenzyme found in boiled extracts of heart and liver. Gale and Epps (18) in their investigations of the bacterial enzymes which decarboxylate amino acids showed that the lysine and tyrosine carboxylases contained a dissociable coenzyme which they called codecarboxylase. They studied the distribution of codecarboxylase, finding that it occurred universally in animals and bacteria. Furthermore, they isolated the coenzyme in highly purified form from yeast. A sample of codecarboxylase prepared from yeast by Gale's

<sup>5</sup> Dopa carboxylase, which was first discovered by Holz *et al.* (17), hitherto has not been obtained in purified and concentrated form. Our method of preparation is as follows. Pig kidneys were finely minced and then extracted with 2 volumes of ice-cold 0.8 per cent sodium chloride solution. After coarse particles were strained out through several layers of wide-meshed gauze, the extract was clarified by centrifugation. The highly opalescent supernatant fluid was then treated with ammonium sulfate (18 gm. per 100 cc.) and the precipitate discarded. Ammonium sulfate was again added (12 gm. per 100 cc.) and the precipitate which contained the enzyme was redissolved in water. The solution of the enzyme was then again submitted to fractional precipitation with ammonium sulfate as above. The solution of the twice fractionated enzyme was dialyzed against 0.002 M ammonia at 0° during 4 days. The solutions so dialyzed are virtually inactive in the absence of codecarboxylase. In addition to the coenzyme, reducing substances such as cysteine or glutathione are essential for reconstituting the activity of the split enzyme. The process of splitting off the coenzyme by dialysis against ammonia water is also attended with considerable irreversible loss of activity. The purification procedure should be carried out as quickly as possible, and always at 0°, to minimize the rate of destruction of the enzyme. The enzyme is most stable when stored as an ammonium sulfate precipitate.

method was found to replace boiled extracts of heart and kidney in restoring the activity of dopa carboxylase. More recently Gunsalus and his colleagues (19) have discovered that pyridoxal which has been subjected to phosphorylating agents, either inorganic or enzymatic, can replace Gale's coenzyme as codecarboxylase for various amino acids and carboxylases of bacterial origin. Through the kindness of Dr. Gunsalus and

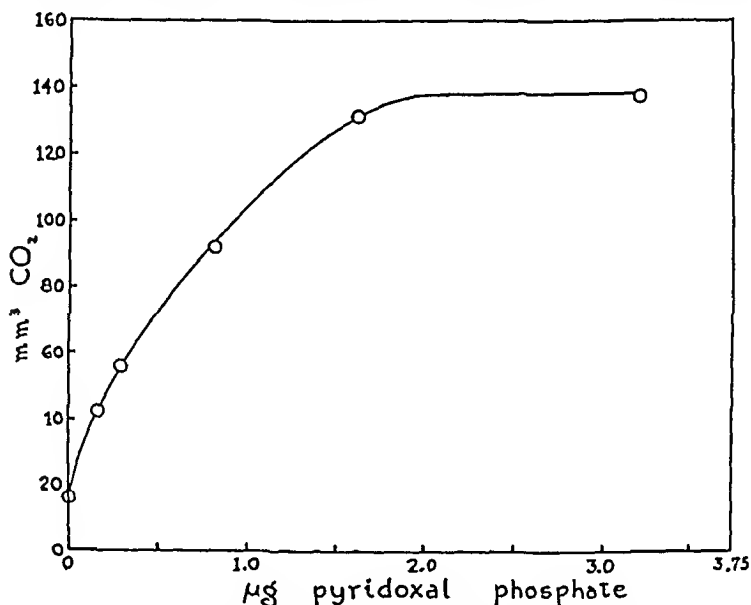


FIG. 6: Rate of decarboxylation of dihydrophenylalanine by dopa carboxylase as a function of the concentration of codecarboxylase. The reaction mixtures contained 0.8 cc. of 0.015 M dihydrophenylalanine, 0.5 cc. of 0.2 M phosphate buffer of pH 7.0, 1 mg. of cysteine, 1 cc. of dialyzed pig kidney enzyme, and varying amounts of synthetic codecarboxylase as prepared by Umbreit *et al.* (19). The gas space was filled with nitrogen. At time zero the substrate was tipped into the central compartment of the manometer cup from a side arm. After 15 minutes 0.4 cc. of 6 N sulfuric acid was tipped in from the center pot and the carbon dioxide evolution was then measured. The  $\text{CO}_2$  evolution was corrected for the blank without enzyme. The reaction was carried out at  $38^\circ$ . The pyridoxal phosphate solution used was not pure, but the concentration in terms of the pure substance has been estimated by Dr. Gunsalus.

Dr. Folkers, we were able to test "pyridoxal phosphate" as coenzyme for dopa carboxylase. It was found to be fully equivalent to Gale's yeast coenzyme in its action on the split dopa enzyme (*cf.* Fig. 6).

The fact that extremely valuable information was obtained by using split *D*-amino acid oxidase as a test system for flavin dinucleotide suggested to us the use of the split dopa enzyme as a test system for codecarboxylase. Various enzyme preparations were tested for the presence of codecar-

boxylase, including the purest preparations of the two transaminases. The extremely high concentration of the codecarboxylase which was found in the solutions of the two transaminases compared to the negligible amounts found in other highly purified enzymes made it seem unlikely that the presence of codecarboxylase was fortuitous. The relationship between the activity of each of the two transaminating enzymes and the amount of codecarboxylase was investigated in different fractions and in the final homogeneous preparations. Table X shows that there is a very satisfactory correlation between the amount of transaminase and the amount of codecarboxylase in the case of the alanine enzyme. The proportionality is admittedly less exact in the case of the aspartic enzyme but there can be no doubt that there is a correlation. The codecar-

TABLE X

*Presence of Codecarboxylase in Preparations of Two Transaminases at Different Stages in Purification*

In testing for codecarboxylase activity the solution of the transaminase was first boiled and then added to the assay system without removing coagulated protein.

Alanine enzyme		Aspartic enzyme	
Purity index	Micrograms codecarboxylase Enzyme units	Purity index	Micrograms codecarboxylase Enzyme units
0.22	0.0037	0.85	0.038
0.10	0.0035	0.55	0.016
0.09	0.0028	0.45	0.018
0.06	0.0042	0.42	0.021
0.034	0.0041	0.14	0.045
0.006	0.0025	0.11	0.032
		0.041	0.023

boxylase associated with the aspartic enzyme is extremely difficult to separate from the coagulated protein and this unpleasant property may well explain some of the variations in the estimation. A crude solution of alanine transaminase after being boiled will show full codecarboxylase activity in the assay system only if the coagulated protein is added. The filtered solution is much less active. As the purification proceeds, the codecarboxylase shows less and less tendency to be retained by the protein coagulum, and at the highest purity level a protein-free solution can be obtained containing the original codecarboxylase activity of the enzyme. In the case of the aspartic enzyme, the tendency of the codecarboxylase to adhere to the coagulated protein is greater and persists from the first crude extracts to the highest purity level. At best a few per cent can be obtained in protein-free solution. The fact that the coagulated protein

shows codecarboxylase activity must mean that the codecarboxylase is at least slightly dissociable, and that it preferentially combines with the protein of dopa carboxylase. In other words, codecarboxylase has a stronger tendency to combine with the protein of dopa carboxylase than with the coagulated protein. However, the transfer may not be quantitative, and that may be at the root of the difficulty in getting better correlations between the amount of codecarboxylase and the amount of aspartic transaminase activity. It is of interest to point out that by boiling "pyridoxal phosphate" in the presence of coagulable protein, the synthetic coenzyme is found to be retained partially or completely in the coagulum to an extent, depending upon the nature and amount of protein added.

Both transaminases lose activity following prolonged dialysis against 0.002 M ammonia at 0°. Thus the alanine enzyme is completely inactive at the end of 3 days. The aspartic enzyme loses activity more slowly, reaching complete inactivation only after about 12 days. This loss in transaminase activity is paralleled by a loss in the codecarboxylase activity of the boiled enzyme. It is not possible to decide which is cause and which is effect; *i.e.*, whether loss in transaminase activity is the result of loss of codecarboxylase or whether inactivation of the enzyme is followed by splitting off of the codecarboxylase. In any case, the two events occur simultaneously and we may regard these dialysis experiments as evidence for the view that codecarboxylase is a functional part of transaminase in the sense of being a coenzyme or prosthetic group.

It became important to know whether the "coenzymes" of the two transaminases are identical with pyridoxal phosphate. Both Gunsalus<sup>6</sup> and Baddiley and Gale (20) have already established the identity of yeast codecarboxylase with pyridoxal phosphate. When the coenzymes were heated for 1 hour at 100°, the per cent destruction of the codecarboxylase activity was found to be 57 for the coenzyme of aspartic transaminase, 65 for the coenzyme of alanine transaminase, and 54 for pyridoxal phosphate, when treated with 0.1 N sulfuric acid; when treated with 0.1 N sodium hydroxide or with neutral solution there was no destruction. There appears to be complete correspondence of the three coenzymes, at least in regard to their pH stability. When the preparations of the coenzyme contain protein as an impurity, it may appear that boiling in neutral solution leads to destruction of the coenzyme. Analysis has shown, however, that this apparent loss of activity is due to a reversible combination of the coenzyme with protein. Exposure to 0.1 N alkali for a few minutes at 100° suffices to regenerate most of the original activity.

The comparison of the three coenzymes was extended to a study of their action in stimulating the growth of *Lactobacillus casei* in a medium lacking in pyridoxine. Table XI shows that all three show growth-

promoting activity at about the same dilutions. Two complicating factors are encountered in testing solutions of coenzyme which contain significant amounts of protein. As pointed out above, the coenzyme shows a strong tendency to combine with protein in the medium when the solution is sterilized by autoclaving. The coenzyme is not available for growth when present in this bound form, but it may be regenerated by hydrolyzing for a short period in 0.1 N HCl at 100°. Some of the coenzyme is hydrolyzed during this period but, since pyridoxal is equivalent to its phosphoric ester as far as growth stimulation is concerned, the estimation is unaffected. When working with a protein-free solution of the coenzyme, no difference

TABLE XI

*Effect of "Coenzymes" of Two Transaminases on Growth of Lactobacillus casei in Pyridoxal-Free Synthetic Medium*

	Micrograms per test	Growth in 48 hrs.
	0	0
Pyridoxal	0.02	+
	0.10	++++
" phosphate	0.005	0
	0.05	++++
"Coenzyme" of alanine-glutamic trans-aminase	0.01	+
	0.05	++++
"Coenzyme" of aspartic-glutamic trans-aminase	0.005	0
	0.05	++++

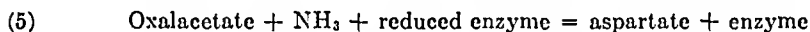
The concentrations of the three coenzymes were determined by comparing their codecarboxylase activity against a standard pyridoxal phosphate solution. The "coenzymes" of the transaminases were prepared by treating the respective enzyme solutions at 100° for 1 hour in 0.1 N acid. The solution of the "coenzymes" was then filtered before testing. The growth medium was that described by Snell and Ranelfeld (21).

in growth-stimulating effects is observed between untreated and acid-hydrolyzed solutions. A second complication which again is referable to the presence of protein in the solution of coenzyme arises from the liberation of growth-stimulating substances by acid hydrolysis of protein. In the absence of pyridoxal these growth-stimulating substances have no influence on the growth of the test organism, but in the presence of pyridoxal they induce a rate of growth which is very much more rapid than in the presence of pyridoxal alone. Thus crude solutions of coenzyme which have been subjected to acid hydrolysis appear even more active than pyridoxal or its phosphoric ester in stimulating growth. However, with purified solutions of the coenzyme containing little or no protein, no significant difference can be discerned between the coenzymes isolated from the two transaminases and pyridoxal phosphate in so far as growth stimulation is concerned.



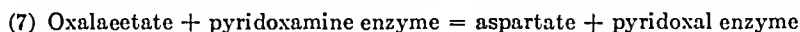
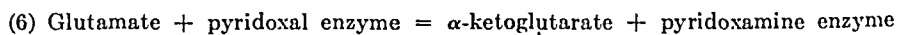
Some as yet unpublished experiments of Gunsalus and his colleagues<sup>6</sup> with *Streptococcus faecalis* have brought evidence from another direction that pyridoxal phosphate is the prosthetic group of transaminase. They grew the organism in a medium deficient in vitamin B<sub>6</sub> and showed that the suspensions of avitaminous bacteria were unable to catalyze the transaminations characteristic of the aspartic and alanine enzymes. However, following addition of pyridoxal phosphate the transaminating activity was completely restored to normal. Schlenk and Snell (22) previously tried to show the participation of pyridoxal in transaminating reactions by adding it to the tissues of vitamin B<sub>6</sub>-deficient rats. However, their experiments were suggestive rather than conclusive.

*Mode of Action of Transaminase*—There are at least three possible mechanisms by which transamination reactions can take place. The reaction between glutamate and oxalacetate may be conceived of as an oxidation-reduction in which glutamate is oxidatively deaminated and oxalacetate simultaneously reductively aminated. According to this view, the process of transamination would be the summation of two separate reactions.



This mechanism can be excluded, since experiments with isotopic ammonia (cf. Shemin<sup>7</sup>) have shown conclusively that free ammonia is not formed during the process of transamination. Furthermore, there is no evidence that transaminase is capable of undergoing reversible oxidation and reduction.

Another mechanism was outlined by Braunshtein and Kritsman (1), according to which the enzyme catalyzed a Schiff base condensation between the amino acid and the keto acid of the transaminating pair. The Schiff base compound would then undergo rearrangement and dissociation into the transaminated pairs. This mechanism was in fact proposed by Herbst (23) to explain transamination reactions in non-biological systems. More recently Snell (24) found that pyridoxal heated with glutamic acid underwent transamination with formation of pyridoxamine and  $\alpha$ -ketoglutaric acid. This led him to postulate that the prosthetic group of transaminase is pyridoxal and that transaminase acted by alternately accepting and donating amino groups, as indicated in the equations below:



There is evidence from our own experiments and those of Gunsalus that the prosthetic group of transaminase is probably pyridoxal phosphate,

<sup>6</sup> Gunsalus, I. C., private communication.

<sup>7</sup> Shemin, D., private communication.

but we have been unable as yet to establish that cyclical amination and deamination of the prosthetic group take place during the process of enzymatic transamination.

It is a pleasure to acknowledge our debt to Miss Marion Blanchard and Dr. P. K. Stumpf for their generous help in studying the properties of the coenzymes obtained from the two transaminases.

#### SUMMARY

The isolation and properties of two transaminating enzymes from pig heart are described. The aspartic-glutamic enzyme catalyzes the transfer of amino groups from oxalacetate to glutamate or from aspartate to  $\alpha$ -ketoglutarate. The alanine-glutamic enzyme catalyzes the transfer of amino groups from pyruvate to glutamate or from alanine to  $\alpha$ -ketoglutarate. Both of these enzymes have been purified to a point at which all the protein present has enzymatic activity. The catalytic constants at this purity level have been evaluated.

Rapid methods have been described for following the action and for determining the amounts of these enzymes. These methods depend upon the appearance or disappearance of oxalacetic acid,  $\alpha$ -ketoglutaric acid, and pyruvic acid.

Evidence has been presented which points to pyridoxal phosphate as the prosthetic group of both transaminating enzymes.

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## ISOLATION OF L-AMINO ACID OXIDASE

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WITH A NOTE BY DAN H. MOORE

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In an earlier communication it was shown that the oxidative deamination of approximately half of the naturally occurring amino acids is catalyzed by a single enzyme, *l*-amino acid oxidase. The general properties of this enzyme isolated from rat kidney and liver, as well as the mechanism of the reactions involved, have already been described (1).

More recently the generosity of several pharmaceutical companies<sup>1</sup> in supplying us with some 50 kilos of rat kidney has made it possible to undertake the isolation of the enzyme. The purification has been brought to a stage at which the enzyme preparation is electrophoretically homogeneous. In the ultracentrifuge two components are found to be present. However, these have been shown to be two molecular species of the enzyme, one having 4 times the molecular weight of the other. The enzyme is a flavoprotein whose prosthetic group has now been identified as the flavin mononucleotide, riboflavin phosphate. The present communication is concerned with the preparation and some of the properties of the purified enzyme and with the evidence for the nature of the prosthetic group.

*Purification of Enzyme*—The purification consists essentially of two successive precipitations of the enzyme with 15 per cent sodium sulfate at pH 5.6, followed by at least three series of controlled stepwise fractionations with ammonium sulfate (*cf.* the experimental section). Since in the early stages of purification many of the impurities are colored compounds, the recognition of the enzyme as a flavoprotein is possible only toward the end. The activity of the final preparation represents at most only a 1 or 2 per cent yield. The over-all purification, starting from the first extract to the final preparation, is about 200-fold. In the early stages considerable losses are sustained at each step but the losses are more than offset by the degree of purification attained.

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<sup>1</sup> We are greatly indebted to Eli Lilly and Company, Hoffmann-La Roche, Inc., Parke, Davis and Company, and Schering-Kahlbaum for generous gifts of frozen rat kidneys.

The preparation of the electrophoretically homogeneous enzyme has a  $Q_{01}$  of 52 in the presence of *L*-leucine at 38° and at pH 8.8. 1 mg. of enzyme at this stage of purity contains 6.6  $\gamma$  of riboflavin phosphate from which it can be calculated that each mole of enzyme catalyzes the oxidation of 6 molecules of substrate per minute under the above conditions. This value for the turnover number is admittedly far below the range for other flavoproteins, which extends from 50 to 1000. The formidable discrepancy in catalytic velocity may indicate any one of three possibilities: (1) that the enzyme is not identical with the flavoprotein, being merely a minute impurity, (2) that individual molecules of the enzyme have undergone "decay" (partial loss of activity) during the purification procedure and that the turnover number of the native unprocessed enzyme would be of the right catalytic order of magnitude, and (3) that *L*-amino acid oxidase is an unusually slow enzyme. The first possibility is inconsistent with the observation that the prosthetic group of the flavoprotein is reduced by amino acids. The actual rate of reduction has been determined and found to be about the same order of magnitude as the over-all oxidation of the substrate by molecular oxygen. This is in accord with the assumption that the flavoprotein is identical with *L*-amino acid oxidase. The flavoprotein is distinct from all other known flavoproteins and, in so far as we have been able to determine, shows no catalytic activity except that characteristic of the *L*-amino acid oxidase. Furthermore it should be pointed out that the final preparation contains only two molecular species both of which are flavoproteins with identical catalytic action. No one of the above points of evidence is in itself adequate, but collectively they leave little doubt of the identity of the *L*-amino acid oxidase with the flavoprotein which has been isolated.

In saying that an enzyme may undergo "decay" during the purification procedure, we employ the term in the sense that single molecules of enzyme may become less active, rather than that a certain proportion of a population of enzyme molecules has become completely inactive, as implied by the term "destruction." There is no single method of deciding whether in the course of purification decay or destruction, as defined above, is proceeding. The fact that there is progressive improvement in the purity index (see "Experimental") argues against any significant amount of decay. Furthermore, in the final stages of purification at which there is no appreciable loss of activity, apart from the partition of the enzyme in different fractions, the process of decay, at least in these stages, can be definitely excluded. It seems likely that the *L*-amino acid oxidase like the vast majority of enzymes exists either as a fully active enzyme or as an inactive enzyme but not in a form that has only partial activity. These considerations exclude all but the third possibility; *viz.*, that the *L*-amino acid oxidase is a very slow acting enzyme.

*Lactic Oxidase Activity*—A strict parallelism has been found between the ability of various preparations from rat kidney to oxidize *l*-amino acids and the ability to oxidize *l*-lactic acid and certain other  $\alpha$ -hydroxy acids.<sup>2</sup> Thus the ratio of these two activities has been found constant from the first crude extracts to the final stage of purification at which the two molecular species of the enzyme are present. Since each of these species is active toward both substrates, it appears highly probable that both activities are associated with the same enzyme. The full description of the lactic enzyme will be reserved for another communication. The turnover number in the presence of lactic acid under optimum conditions of substrate and hydrogen ion concentrations is approximately 2.7 times as large as that in the presence of *l*-leucine. The flavoprotein is bleached by lactic acid just as it is by *l*-amino acids and the autoxidation of the leucoflavoprotein explains the formation of  $H_2O_2$  during the catalytic oxidation of lactic acid in the presence of molecular oxygen.

*Absorption Spectrum of Enzyme*—Flavoproteins have been shown to have an absorption spectrum that differs only in minor respects from that of free riboflavin in the region 320 to 520  $m\mu$  (3-5). The curve<sup>3</sup> for *l*-amino acid oxidase shown in Fig. 1 displays a maximum in the region of 450  $m\mu$ , which is typical, but the band is broader than that of riboflavin, since it extends to 410  $m\mu$  before declining. The second band of the oxidase, as expected, has a maximum at 360  $m\mu$ , but the extinction coefficient is higher than expected in relation to that of the 450  $m\mu$  band. It is apparent that a second light-absorbing substance is present which, for reasons to be discussed below, we consider to be an impurity present in low concentration but possessing a very high extinction coefficient in the region of 410  $m\mu$ . As purification proceeds, the amount of absorption due to this substance grows progressively smaller. Removal of the final traces entails larger loss than is possible to take with the small quantities of purified enzyme which have been at our disposal.

On reduction with hydrosulfite 75 per cent of the color is bleached at 460  $m\mu$ , the degree of bleaching becoming less at shorter wave-lengths, as shown in Fig. 1. The plot of the difference between the curves for the oxidized and reduced enzymes (the  $\Delta$  curve) does not represent exactly the curve of the flavin component of the protein, because the impurity has also undergone change in the presence of hydrosulfite. The inflection at 410  $m\mu$

<sup>2</sup> In a preliminary report (2) we erroneously referred to the second catalytic function of *l*-amino acid oxidase as a diaphorase action. The test system devised for detecting diaphorase activity contained lactic acid. The positive results we obtained were due to the fact that *l*-amino acid oxidase oxidized lactic acid directly and not to the ability of the enzyme to function as a diaphorase.

<sup>3</sup>  $\epsilon = 1/cl \log_{10} I_0/I_x$ ;  $c$  = the concentration in moles per liter and  $l$  = the cell length in cm.

which appears in both the  $\Delta$  curve and that for oxidized enzyme strongly suggests that the contaminating impurity is of an iron-porphyrin nature. On reduction, iron-porphyrin proteins generally show an increased absorp-

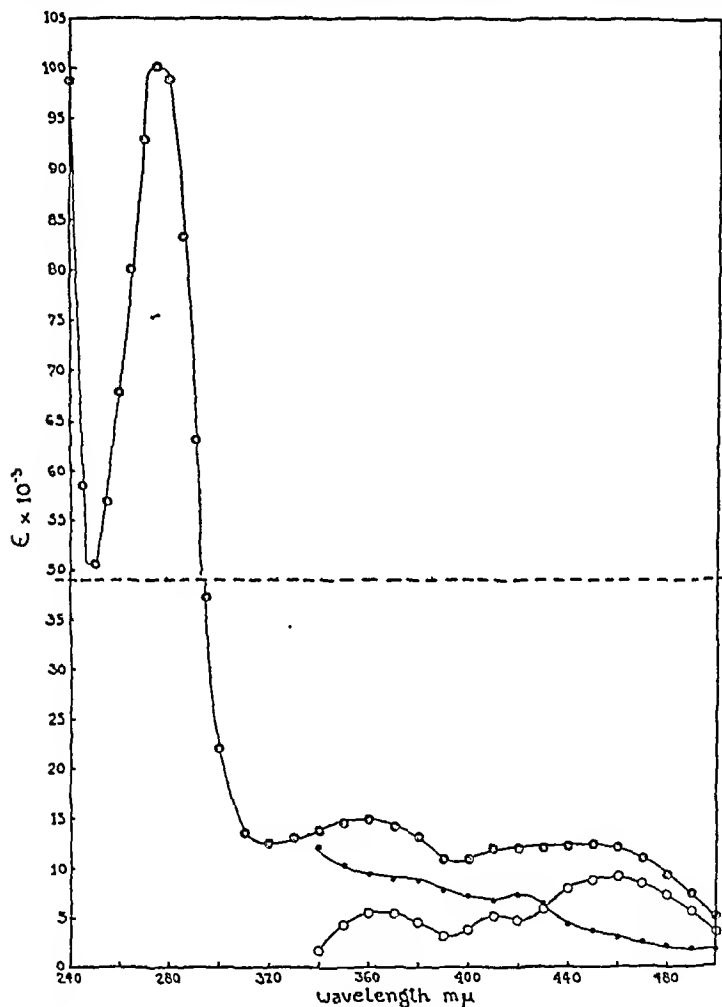


FIG. 1. Light absorption spectrum of *L*-amino acid oxidase in 0.1 M phosphate buffer at pH 7.4. Double circles represent oxidized enzyme; solid circles, enzyme reduced with hydrosulfite; clear circles, the plot of the difference between the absorptions of the oxidized and reduced enzyme.

tion and slight shift of the Soret band, while absorption in the regions to either side may be increased at some wave-lengths and decreased at others.<sup>4</sup>

<sup>4</sup> Compare, for example, the light absorption curves of ferri- and ferrocytochrome *c* reported by Theorell (6).

This property would account for the fact that the  $\Delta$  curve varies in some respects from that expected for the flavin component. Furthermore, iron-porphyrin compounds have in common the Soret band in the region of 410  $m\mu$  with an extremely high extinction coefficient. For example, the value<sup>5</sup> given by Theorell and Åkesson (7) for cytochrome *c*,  $\epsilon_{410} = 118 \times 10^3$ , is 26 times greater than that of riboflavin at the same wave-length. This factor is increased to 137 when the molecular weights are taken into account, cytochrome *c* having a molecular weight of 13,100 as compared to 69,000 for the minimum molecular weight of *l*-amino acid oxidase. Therefore in order to account for the absorption of the oxidized enzyme at 410  $m\mu$ , which is double that expected of a flavoprotein, no more than 0.7 per cent of cytochrome *c* or an equivalent amount of some other iron-porphyrin protein would have to be present as a contaminant of the *l*-amino acid oxidase. This amount of impurity is less than can be detected electrophoretically. The fact that the enzyme appears homogeneous in the Tiselius apparatus but displays an impurity on spectrophotometric examination permits the conclusion that the impurity is present in very low concentration and is probably an iron-porphyrin compound. It was not surprising to encounter the problem of removing such impurities during isolation of flavoproteins from animal tissue, in which the concentration of iron-porphyrin compounds is extremely high in relation to flavoproteins. Warburg and Christian (8) found a similar contaminant of the "old yellow enzyme" isolated from yeast.

The flavin phosphate content of the enzyme is 0.66 per cent. This value was obtained spectrophotometrically by splitting the flavoprotein with trichloroacetic acid and removing the denatured protein by centrifugation. The colored impurity is precipitated with the protein in this procedure (see the section on the prosthetic group), thus permitting an accurate estimate of the flavin content. Since the data for the curves shown in Fig. 1 were calculated from the actual flavin phosphate content of the enzyme and the observed optical density, it may be seen that at 460  $m\mu$  the impurity has the effect of elevating the absorption by 17 per cent, as compared with the value given by Theorell (3) for the "old yellow enzyme,"  $\epsilon_{460} = 10.4 \times 10^3$ . It is also apparent from Fig. 1 that the difference between the oxidized and reduced values,  $\Delta\epsilon_{460}$  may not be used for accurate estimation of the flavin content of the enzyme, since it represents a summation of the changes undergone by both the flavin and the iron-porphyrin impurity. The value for  $\Delta\epsilon_{460}$  is actually 12 per cent lower than would be expected on the basis of the known flavin content.

<sup>5</sup> The original value was expressed as  $\beta$ , the molecular absorption constant, and has been converted to the  $\epsilon$  value in order to facilitate comparison.



### Identification of Prosthetic Group

**Absorption Spectrum of Prosthetic Group**—The losses sustained in the purification procedure are such that 3 kilos of fresh rat kidney furnish only about 5 cc. of a 1.5 per cent solution of the purified enzyme. Owing to this restriction, identification of the prosthetic group was established by employing enzyme preparations only of the highest purity level, as an alternative to direct isolation. The prosthetic group does not dissociate from the protein to any appreciable degree and cannot, therefore, be recovered by mild procedures. When a solution of the enzyme is denatured by exposure to a temperature of  $100^{\circ}$ , the prosthetic group is split off but simultaneously undergoes considerable destruction. However, if the enzyme solution is brought to dryness rapidly in an oven at  $105^{\circ}$  and the thin film of protein extracted with small amounts of water, the recovery of flavin phosphate is

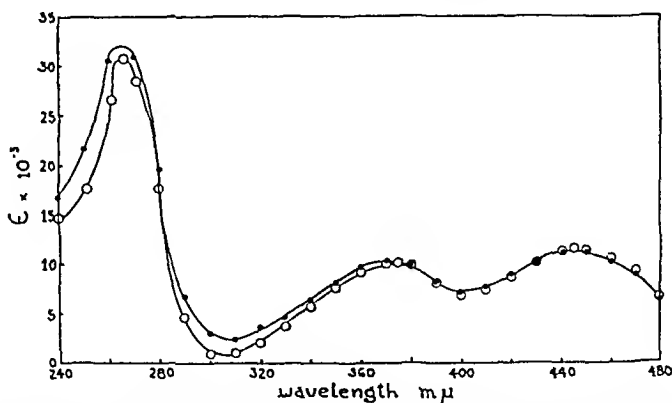


FIG. 2. Light absorption spectrum of the prosthetic group of *L*-amino acid oxidase (solid circles) and of riboflavin (clear circles). Aqueous solutions at pH 7.0.

fair and the decomposition is negligible. The extract is yellow, with the characteristic greenish fluorescence of riboflavin. This material, without further treatment, was used to obtain the light absorption curve shown in Fig. 2. The curve for free riboflavin (Merck) is also presented for comparison. The flavin concentration of the solution of the prosthetic group was calculated from the observed optical density at  $450\text{ m}\mu$  and the extinction coefficient,  $\epsilon_{450} = 11.3 \times 10^3$ , as given by Warburg and Christian (9) for riboflavin or riboflavin phosphate.<sup>5</sup> It may be seen that the two curves are practically superimposable with characteristic maxima at  $450$ ,  $375$ , and  $265\text{ m}\mu$ .

The molecular light absorption curves of riboflavin and its phosphoric ester are coincident over the range investigated. However, the absorption curve of flavin adenine dinucleotide represents the summation of the absorption due respectively to riboflavin and adenine (9). Since adenine

does not absorb appreciably at wave-lengths greater than 300  $m\mu$ , the curve for the dinucleotide is virtually identical with that of riboflavin down to 300  $m\mu$ . At 265  $m\mu$ ,  $\epsilon$  for riboflavin is  $29.4 \times 10^3$ , while that for the dinucleotide is  $37.0 \times 10^3$ . As shown in Fig. 2, the  $\epsilon$  value for Merck's riboflavin,  $30.5 \times 10^3$ , is in good agreement with the value set by Warburg and Christian (9), while the  $\epsilon$  value for the prosthetic group is  $32.0 \times 10^3$ . The presence of a second absorbing component, either purine or pyrimidine in nature, would have increased the  $\epsilon$  value of the prosthetic group to  $37 \times 10^3$  or higher, since such compounds all have absorption coefficients of about the same order of magnitude as adenine itself. The slightly higher absorption of the prosthetic group observed in the region of shorter wave-length compared to that of riboflavin may justifiably be attributed to the presence of traces of impurities formed during the heat treatment. Apparently the riboflavin of *l*-amino acid oxidase is not associated with a second light-absorbing molecule, as would be expected were the prosthetic group a dinucleotide. The same absorption curve is obtained when the prosthetic group is split off from the protein following denaturation with trichloroacetic acid, after suitable correction for the pH effect and light absorption due to the reagent.

*Ratio of Phosphorus to Flavin in Prosthetic Group*—The prosthetic groups of all the flavoproteins known thus far occur either as riboflavin phosphate or as flavin adenine dinucleotide. In order to distinguish between these two possibilities, advantage has been taken of (1) the differences in absorption at 265  $m\mu$ , as already discussed above, and (2) the difference in the ratio of P to flavin, the mononucleotide and the dinucleotide having ratios respectively of 1:1 and 2:1.

The P to flavin ratios were determined on samples of the prosthetic group prepared in several different ways, as indicated in Table I. The flavin content of Preparation 1 was estimated spectrophotometrically assuming  $\epsilon = 11.3 \times 10^3$  at 450  $m\mu$  (9). This value is only applicable to neutral solutions. In strong acid, the absorption of riboflavin is decreased in the region of the first maximum, and increased in the region of the second. A value of  $\epsilon_{450} = 9.8 \times 10^3$ , obtained from flavin solutions of known concentration in 4.1 per cent trichloroacetic acid, has been used to estimate the flavin phosphate content of enzyme solutions to which trichloroacetic acid was added as the protein precipitant (Preparations 2 to 5). The light absorption of trichloroacetic acid is very small in this region and was adequately corrected for by a suitable blank.

In order to estimate the P content of these solutions, a submicroprocedure (see "Experimental") having a range of 0.2 to 3  $\gamma$  of P was employed. It was found necessary to estimate both inorganic and organically bound P, since even after prolonged dialysis traces of inorganic P were still present.

All of the samples investigated, as shown in Table I, were found to have a ratio of organic P to flavin of 1:1. This was the case regardless of the treatment employed and serves to confirm the evidence, obtained from the absorption spectrum, that the prosthetic group is riboflavin phosphate.

It should be pointed out that on prolonged dialysis there is some loss of flavin, for Preparation 5 which was not dialyzed has the highest flavin content. The simultaneous loss of an equivalent amount of organic P appears to indicate that on dialysis the prosthetic group is split from the protein at the rate of a few per cent a day, without cleavage of the ribityl phosphoric

TABLE I  
*Ratio of Organic P to Riboflavin in l-Amino Acid Oxidase*

Preparation No.	Treatment	Riboflavin <i>γ</i> per cc.	Inorganic phosphorus <i>γ</i> per cc.	Organic phosphorus* <i>γ</i> per cc.	Molecular ratio of organic P to riboflavin
1	Dialyzed 7 days against distilled water, oven-dried	41.3		3.32	1.00:1.00
2	Dialyzed 7 days against distilled water, treated with trichloroacetic acid	50.3	0.63	4.81	1.00:0.86
3	Dialyzed 6 days against distilled water, treated as Preparation 2	55.9	1.74	4.23	1.00:1.08
4	Dialyzed 6 days against distilled water brought to pH 8 with NH <sub>4</sub> OH, treated as Preparation 2	43.9	2.54	3.41	1.00:1.06
5	Undialyzed; treated as Preparation 2	77.8	5.64	6.36	1.00:1.01

\* The figures in this column represent the difference between total and inorganic phosphorus.

ester linkage. Presumably the splitting is responsible for inactivation of the enzyme and it explains why dialysis is invariably associated with loss of catalytic activity.

The theoretical possibility remains that the prosthetic group of *l*-amino acid oxidase may exist as a conjugate of two riboflavin phosphate nucleotides, although as yet no naturally occurring examples of such a configuration are known. It is to be expected that a compound of this nature will be much less acidic than riboflavin phosphate. Investigation of this property must await isolation.

*Number of Flavins per Molecule*—In an earlier publication (2) it was reported that the preparation of purified enzyme was found to be homogeneous when examined electrophoretically at pH 7.4. In the ultra-

centrifuge, two molecular species of the flavoprotein were found to be present, 60 per cent as a lighter form, having a molecular weight of 120,000, and 40 per cent of a heavy fraction which may be regarded as an aggregate of 4 of the lighter molecules.

In the same communication the flavin content was reported as 0.46 per cent, expressed as riboflavin phosphate. Since this corresponds to a minimum molecular weight of 100,000 as compared to 120,000 obtained from ultracentrifuge data, the enzyme was regarded provisionally as having one riboflavin phosphate per molecule of protein. The estimation of the flavin content was carried out in samples obtained by extraction of oven-dried samples previously subjected to prolonged dialysis to insure complete removal of ammonium sulfate. We have since observed that the prosthetic group is split off at the rate of a few per cent a day when dialyzed against distilled water. This loss of flavin, as well as incomplete recovery from the dried film, led to erroneously low results. When the flavin content of undialyzed enzyme preparations was estimated by direct spectroscopic measurement of the supernatant fluid, after precipitation of the protein with trichloroacetic acid at 0°, the value was found to be 0.66 per cent, expressed as riboflavin phosphate, corresponding to a minimum molecular weight of 69,200. It is extremely unlikely that a value appreciably higher than 0.66 per cent will be obtained, since the entire purification procedure includes but one dialysis for 18 hours. The value of 0.66 per cent therefore strongly suggests that there are two riboflavin phosphate groups for each protein unit of approximately 138,000. Moore's value (see the appended note) of 120,000 is in substantial agreement with this interpretation. The heavy component would then have a molecular weight of 552,000, containing eight flavin groups in all.

#### EXPERIMENTAL

*Methods of Following Activity*—We have arbitrarily defined as the unit of *l*-amino acid oxidase activity an amount of enzyme which in the presence of excess *l*-leucine and excess catalase<sup>6</sup> at pH 8.8 and 38° catalyzes the uptake of 60 c.mm. of O<sub>2</sub> per hour. All solutions of the enzymes, substrates, and buffer are previously adjusted to pH 8.8. To the enzyme solution in a Warburg vessel are then added 0.3 cc. of catalase and 0.5 M phosphate buffer to make a final volume of 3.0 cc.; 0.5 cc. of 0.1 M *l*-leucine in 0.5 M phosphate buffer is tipped in from the side arm after temperature equilibration, and the oxygen consumption estimated manometrically. Since cata-

<sup>6</sup> When excess catalase is present, exactly 1 atom of oxygen per molecule of substrate will be consumed (1). The catalase solution employed was a concentrated preparation obtained from pig kidney extract by two precipitations with 30 per cent ammonium sulfate, followed by dialysis.

lase is largely destroyed during Step 3 of the isolation procedure given below, it must be added to the test run.

As a rapid and convenient measure of the degree of purity of the enzyme at each step of the purification, we have employed the purity index defined as the ratio of the optical density at  $280\text{ m}\mu$  in a 1 cm. cuvette to the number of units per cc. of enzyme. As the purification proceeds, the purity index becomes smaller, which means in effect that the amount of protein per unit of enzyme becomes progressively less until all the protein is associated with the enzyme itself. Because of the fact that ammonium sulfate inhibits *l*-amino acid oxidase even when present in concentrations as low as  $0.01\text{ M}$ , it is not possible to test directly for *l*-amino acid oxidase activity during ammonium sulfate fractionations. The solutions might be dialyzed to remove the ammonium ion but apart from considerable delay there is the destruction of the enzyme which invariably attends dialysis. This difficulty has been overcome by taking advantage of the fact that the *l*-amino acid oxidase also oxidizes  $\alpha$ -hydroxy acids such as lactic acid. The ratio, rate of oxidation of *l*-leucine to rate of oxidation of *l*-lactate, has repeatedly been found to be 1:2.7. The standard conditions employed for estimating the oxidation of lactic acid are identical with those given above for *l*-leucine except for the substitution of substrates. The oxidation of lactic acid is not inhibited by ammonium ions. Thus by estimating the oxidation of *l*-lactic acid it becomes possible to assay the *l* enzyme accurately in the presence of ammonium sulfate.

In the final stages of the purification, when it is of the utmost importance to conserve material, one can avoid using up samples in a catalytic test by substituting a spectrophotometric index of purity. The iron-porphyrin impurity markedly increases the absorption at  $410\text{ m}\mu$  without influencing as appreciably the  $450\text{ m}\mu$  band characteristic of flavin. The ratio of extinctions,  $\epsilon_{410}/\epsilon_{450}$ , becomes therefore a measure of the degree of purification of the flavoprotein as the limiting value of 0.5 is approached. The best ratio we have reached has a value of 0.96, whereas in cruder fractions the value of the ratio may be 10 to 20 times as high. The purity index and the extinction ratio have been applied only as rapid and convenient measures for following the purification. The catalytic activity and flavin phosphate contents of the purified enzyme are always based on a direct estimation of the dry weight of the protein.

### *Preparation of Enzyme*

*Step 1*—Rat kidneys (about 1.5 kilos) were minced in a Waring blender to a fine cream and poured into 4 volumes of acetone cooled to  $-5^\circ$  with dry ice. The suspension was rapidly filtered with suction and the residue washed several times with cold acetone. The residue was powdered, al-

lowed to dry in air, then resuspended in acetone (room temperature) to remove as much of the residual fat as possible, filtered, and again allowed to dry in air. 318 gm. of dry powder were obtained.

*Step 2*—The kidney powder was mixed with 10 volumes of water and the suspension allowed to stand for 30 minutes with constant stirring. The insoluble particles were filtered off and washed thoroughly with water. The volume of the combined filtrates was about 3.9 liters, containing 347 units with a purity index of 347. Anhydrous sodium sulfate was added (15 gm. per 100 cc. of solution) and then glacial acetic acid to bring the pH to 5.1, as measured electrometrically with a glass electrode. The precipitate was filtered through fluted papers. The filtration is rather slow and may be safely carried on overnight if necessary. The enzyme is quite stable in this precipitate.

*Step 3*—The precipitate was suspended in water and 6 N NaOH was added to bring the pH to 8.5. The final volume was 360 cc., containing 222 units. The suspension was heated to 57° for 5 minutes and the bulky coagulum filtered off. The filtrate (310 cc. containing 85 units) was treated again with sodium sulfate (15 gm. per 100 cc.), the pH being brought to 5.6 with 10 per cent acetic acid. The adjustment of pH is critical and should be carried out electrometrically. The precipitate was centrifuged off, resuspended in water (50 cc.), and the pH brought to 8.7 with 6 N sodium hydroxide. The insoluble residue was centrifuged off and discarded. The clear supernatant fluid (56 cc.) then contained 48 units with a purity index of 13.9.

*Step 4*—The above enzyme solution was dialyzed for 18 hours at 0° against 3 liters of 0.025 M phosphate buffer of pH 7.3. The pH of the ice-cold dialyzed solution was cautiously brought to 4.9 with 10 per cent acetic acid. The precipitate containing the bulk of the hematin impurities was discarded. The supernatant fluid, which from this step on has the greenish yellow appearance of flavin solutions, was neutralized with 6 N sodium hydroxide.

*Step 5*—The enzyme solution was then subjected to a series of stepwise fractionations with ammonium sulfate between 30 and 60 per cent saturation. Table II is typical of the results with the first series of fractionations. When Fractions II and III of this stepwise fractionation were combined and refractionated, a second series of fractions with the following characteristics was obtained: Fraction A, 4.8 total units with a purity index of 11.8; Fraction B, 12.4 total units with a purity index of 3.1; Fraction C, 10.4 total units with a purity index of 3.1; and Fraction D, 2.0 total units with a purity index of 15.0. Fractions B and C were combined and refractionated in a third series in which the ratio of 410:450  $m\mu$  was Fraction b, 1.18; Fraction c, 0.96; Fraction d, 1.17. Fraction a consisted of a

solution too opalescent for measurement, denatured material largely. Fraction c contained 4.8 units in 2 cc. with a purity index of 1.7. Solutions of the enzyme at this stage of purity appeared homogeneous in the Tiselius apparatus and were used for spectrophotometric analysis.

In order to effect a separation of the light and heavy molecular species for examination of catalytic activity, a sample of the purified enzyme was treated in the ultracentrifuge as described in the note by Moore. To conserve material the enzyme content of each fraction was determined by spectrometric estimation of riboflavin phosphate. One fraction (the light component, containing 5 per cent of the heavy one) had 0.44 unit of catalytic activity per microgram of riboflavin phosphate. A second fraction (the heavy component containing a small amount of the light one) had 0.40 unit per microgram.

TABLE II  
*Stepwise Fractionation of Enzyme Solution*

Fraction No.	Per cent saturation of ammonium sulfate	Total units*	Purity index*
I	36	3.4	19.3
II	43	20.4	10.4
III	50	15.2	10.1
IV	58	2.9	22.2

\* As defined in the "Experimental."

*Estimation of Total Phosphorus*—The procedure described is a micro adaptation of the method of King (10) in that perchloric acid is used for digestion and the color is developed in a stronger acid than employed originally by Fiske and Subbarow (11), but it incorporates several valuable features employed by Bessey and Lowry (12) in their submicromethod. These are the development of color at an elevated temperature (here 100°) to increase sensitivity, and measurement of the light absorption at 820 m $\mu$ .

With the Beckman spectrophotometer and standard 1 cm. cuvettes, phosphorus in the range of 0.2 to 3  $\gamma$  can be estimated with an accuracy of 2 per cent. Trichloroacetic acid does not interfere with the estimation. The digestion tubes, which were also used for the final color development, were made of Pyrex tubing of 7 mm. internal diameter blown out to a bulb of 20 mm. outside diameter and having an over-all height of about 165 mm. These were calibrated to contain 3.5 cc., the bulb size being selected to bring the calibration mark from 5 to 10 mm., but no higher, above the neck of the bulb. Samples having a volume no greater than 2.5 cc., to which 0.4 cc. of 72 per cent perchloric acid has been added, were digested in an oil bath at 200° for about 15 minutes after bringing the bath

up to this temperature over a 20 minute interval. The slow rise in temperature insured smooth distillation of water and of trichloroacetic acid which were completely removed by the time 200° was reached. Digestion then proceeded without bumping and no boiling stones were required. For most compounds, including phospholipids, this digestion time was sufficient but it may, without introduction of errors, be increased to an hour or longer if necessary.

After cooling the tubes, first 3.0 cc. of 0.33 per cent ammonium molybdate (2 cc. of the stock 5 per cent solution diluted to 30 cc.) were added, and then, after thorough mixing, 0.1 cc. of the aminonaphtholsulfonic acid reagent prepared according to King (10). A few hundredths of a cc. of perchloric acid distilled off during digestion, making necessary a final adjustment of the volume to 3.5 cc. with water. The contents were mixed very thoroughly again with a thin rod flattened at the end. The color was developed by heating in a boiling water bath for 10 minutes. A straight line was obtained for the standard curve; 1  $\gamma$  gives rise to an optical density of 0.255 when read at 820  $m\mu$  in 1 cm. cuvettes against blanks similarly treated.

*Inorganic Phosphate*—In order to estimate inorganic phosphate in a solution containing riboflavin phosphate, without sacrificing sensitivity, it was considered desirable to reduce to a minimum the concentration of acid as used in the above estimation of total phosphorus in order to prevent hydrolysis of the phosphoric ester during color development at 100°. The concentration of acid and of the other reagents employed by Fiske and Subbarow (11) for inorganic phosphate was therefore scaled down to a submicro level. Samples having a volume no greater than 2.75 cc. when trichloroacetic acid was present, or 1 cc. less when it was absent, were transferred to the same tubes as used above, followed by 0.6 cc. of 3  $N$   $H_2SO_4$ . If 1 cc. of 5 per cent trichloroacetic acid, or the equivalent acidity was not included in the sample, it was added to insure the final required acidity. If necessary, water was then added to a volume of about 3 cc. with thorough mixing. This was followed by the addition of 0.1 cc. of 5 per cent ammonium molybdate and finally by 0.05 cc. of the aminonaphtholsulfonic acid reagent used above. The volume was brought to 3.5 cc. Thorough mixing after the addition of each reagent, the final mixing being effected with a stirring rod, is absolutely essential to insure reproducible results. The tubes were then immersed in a boiling water bath for 10 minutes and the density estimated as described above; the density increased by 0.250 for each microgram of increment.

The estimation of inorganic phosphate at the low acidity employed was occasionally troublesome, owing to some variation in the blanks. This was avoided by very careful and thorough mixing after the addition of



each reagent and by checking on the agreement of duplicate or triplicate blanks. When hydrolysis of organically bound phosphorus is not a complicating factor, the conditions described above for total phosphorus, but employed without digestion at 200°, provide a more satisfactory procedure for the rapid estimation of very small amounts of inorganic phosphate.

*Preparation of Samples for Estimation of Flavin and Phosphorus*—1 cc. aliquots of a purified enzyme preparation containing 14.27 mg. of enzyme and 94.3 mg. of riboflavin phosphate per cc. were dialyzed against distilled water for various periods, transferred quantitatively to a 5 cc. volumetric flask, and made up to volume. To each was added 1 cc. of 50 per cent trichloroacetic acid in the cold and the mixture centrifuged at 5° on the high speed head of the International centrifuge. Total P was estimated on 0.5 cc. and inorganic P on 1 cc. aliquots of the supernatant. For estimation of flavin in the Beckman spectrophotometer, 2 cc. were diluted with an equal volume of water to reduce the trichloroacetic acid concentration to 4.1 per cent. Other dialyzed samples were rapidly brought to dryness in an oven at 105°, extracted with small portions of water, filtered, and brought to 5 cc. Solutions of the prosthetic group obtained by this treatment were used for P and flavin analysis as well as to provide the data given in Fig. 1. The curve agrees throughout with the absorption curve obtained, after suitable correction for the pH effect and light absorption of the reagent, from solutions in which trichloroacetic acid had been used to precipitate the protein.

The values for riboflavin phosphate, inorganic phosphorus, and organic phosphorus are given in Table I. Except for the case of the undialyzed sample, inorganic phosphorus was present in very small amounts (0.1 to 0.2  $\gamma$  per sample) and could not be estimated with the same accuracy as the total phosphorus. The uniformity of the ratios in the last column of Table I indicates that riboflavin is present as a phosphoric ester which undergoes no appreciable hydrolysis under the conditions for estimating inorganic phosphate. This was to be expected from the hydrolysis rates of riboflavin phosphate reported by Kuhn and Rudy (13), who found only 22 per cent hydrolysis after boiling for 2 hours in 15 per cent HCl.

#### SUMMARY

1. L-Amino acid oxidase has been shown to be a flavoprotein with riboflavin phosphate as the prosthetic group.

2. By an extensive series of salt fractionations, the enzyme has been brought to a stage at which it is electrophoretically homogeneous. In the ultracentrifuge, two components appear, having identical catalytic activity. The evidence suggests that these are two molecular species of the same enzyme, the heavy component being an aggregate of 4 molecules of the light one.

3. On the basis of the flavin content of the enzyme (0.66 per cent expressed as riboflavin phosphate) and other data, it appears that the light component contains 2 molecules of flavin, whereas the heavy one contains 8.

4. From the parallelism between the ability to oxidize *l*-amino acids and *l*- $\alpha$ -hydroxy acids, it is concluded that the flavoprotein which has been isolated possesses both these catalytic activities.

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#### *Examination of l-Amino Acid Oxidase by Electrophoresis and the Ultracentrifuge*

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The preparation was examined electrophoretically in 0.02 M sodium phosphate buffer of pH 7.4, which was 0.15 M with respect to sodium chloride, and found to be essentially homogeneous. The electrophoresis pattern of the purified preparation is shown in Fig. A. When examined in the same solvent in the ultracentrifuge, however, two components appeared, a heavier fraction constituting about 60 per cent of the total protein ( $S_{20} = 13.5$ ) and a lighter fraction having  $S_{20} = 5.0$ . A trace of heavier material ( $S_{20} = 18$ ) appears in the pattern (Fig. B).

In order to obtain more information about the molecular weight of the components and to determine whether enzymatic activity was associated with one or both molecular species, a 30 cc. portion was centrifuged in eight tubes at 48,000 R.P.M. for 45 minutes. At the speed selected, most of the heavy component and only a small fraction of the light one would sedi-

mented in 45 minutes. 22 cc. of the supernatant were carefully removed. This supernatant was richer in the lighter fraction, whereas the pellet and the concentrated fluid at the bottom of the tubes contained a higher proportion of the heavy fractions than the original. This general procedure was repeated three times, whereupon the supernatant was found to contain less than 5 per cent of the heavy fraction when reexamined in the analytical ultracentrifuge. The pellets from the first of the above centrifugations were analyzed for enzymatic activity. It was found that the separated light fraction and the redissolved pellets (enriched in heavy fraction) contained the same enzymatic activity per weight of protein.

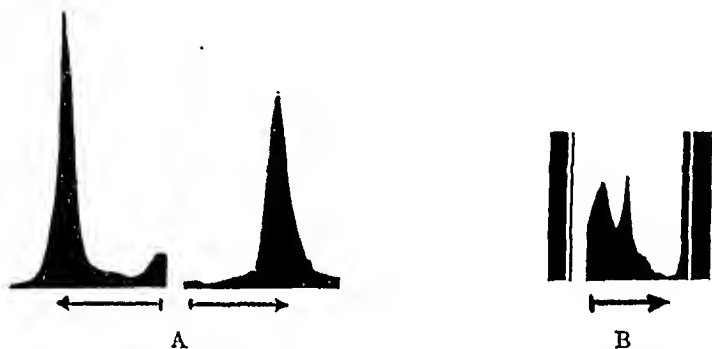


FIG. A. Electrophoresis pattern of *L*-amino acid oxidase. Left, ascending; right, descending. Buffer, 0.02 *M* sodium phosphate in 0.15 *M* sodium chloride, pH 7.4.

FIG. B. Ultracentrifuge pattern of *L*-amino acid oxidase after 40 minutes at 48,000 R.P.M. Protein concentration 0.4 per cent; solvent, same as for electrophoresis.

The supernatant (heavy component-free) solution was allowed to diffuse in the Tiselius electrophoresis cell at 1°. The diffusion constant when corrected to 20° was calculated to be  $4.0 \times 10^{-7}$  sq. cm. per second. Assuming a partial specific volume of 0.75, a molecular weight of about 120,000 was calculated. An accurate estimate of the diffusion constant of the heavy component cannot be made, owing to the impossibility of preparing a sample entirely free of the light component. The average diffusion rate (calculated from the width of the boundary pattern at the inflection point) of the original unseparated material was found to be  $3.0 \times 10^{-7}$  sq. cm. per second. This value is erroneously high owing to the presence of the light component and will result in an estimate of the molecular weight which is correspondingly low. With  $D_{20} = 3.0$  and  $S_{20} = 13.5$  a molecular weight of 430,000 was calculated. Since this value is admittedly low, it appears more probable that the heavy component is an aggregate of 4 molecules of the light component.

# THE QUANTITATIVE DETERMINATION OF THYROXINE IN IODINATED CASEIN HAVING THYROIDAL ACTIVITY\*

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As a result of recent investigations (reviewed by Reineke and Turner (1)) methods are now established whereby iodinated proteins possessing high thyroidal activity, as determined by biological assay, can be formed. The isolation of crystalline *dl*-thyroxine from iodinated casein subsequent to alkaline hydrolysis (2-4) and of the natural *l* isomer after hydrolysis with acid (5) provides good evidence that the active principle is actually thyroxine. Because of serious questions as to the specificity, when applied to iodinated proteins, of the chemical methods for determining thyroxine in thyroid tissue it has been necessary to depend on biological methods for quantitative estimates of the potency of such preparations.

The use of *n*-butanol for selective extraction of thyroxine from hydrolysates of thyroid tissue was introduced by Leland and Foster (6). Blau (7) reported that more complete extraction of thyroxine was effected when the initial step was carried out in an aqueous acid medium instead of in the sodium hydroxide solution used in the original method. He also introduced an alkaline washing solution that provided a more favorable distribution ratio for removal of non-thyroxine iodine compounds from the *n*-butanol extract. In a second communication Blau (8) reported that the thyroxine could be extracted selectively from thyroid tissue after only 6 hours hydrolysis in 8 per cent barium hydroxide, thus avoiding the losses encountered during the longer hydrolysis with 2 *N* sodium hydroxide.

In the present report the thyroxine values obtained on iodinated proteins by biological assay and by a chemical extraction procedure have been compared. While Blau's original method appears not to be entirely specific for thyroxine when applied to iodinated proteins, a modification is introduced that yields values which compare very favorably with those obtained by biological assays.

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## EXPERIMENTAL

The first analyses were made on a series of preparations described previously by Reineke, Williamson, and Turner (9) in which the amount of iodine added during the iodination was increased progressively in the successive preparations. The procedure was similar to that described by Blau (8) except that a smaller portion of iodinated casein was taken for the final analysis because of its higher thyroxine content compared to that of thyroid tissue.

*Hydrolysis in 8 Per Cent Barium Hydroxide*—1.0 gm. of iodinated casein, 3.2 gm. of  $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ , and 2 ml. of *n*-butanol were mixed with 50 ml. of distilled water in a 125 ml. Erlenmeyer flask and boiled gently under a reflux on a hot-plate for 6 hours. The hydrolysates were transferred to volumetric flasks, acidified with dilute hydrochloric acid, and made up to 100 ml. Aliquots containing 25 ml. of this hydrolysate were then extracted and analyzed for *n*-butanol-soluble iodine as in the Blau procedure.

The assays on tadpoles, expressed as the percentage response produced by each preparation compared to a *dl*-thyroxine standard, are taken from the previous report (9). Since we have found that iodinated proteins consistently produce a greater effect on tadpoles than would be expected from their stimulation of metabolic rate in guinea pigs, the data on tadpoles are not believed to provide an absolute measure of thyroxine content. They do, however, indicate the relative thyroidal potency of each preparation compared to that of other members of the series.

When compared with the tadpole assays (Fig. 1), the thyroxine values obtained by the chemical procedure designed for thyroid tissue follow the same general course on preparations of increasing iodine content until the point of optimum iodination or maximum thyroidal potency is reached. Thereafter, the analytical values show only a small decrease, whereas the biological assay indicates an abrupt decline in activity. The apparent thyroxine content ranged from 1.39 per cent in the least active to 3.0 per cent in the most active preparation of the series.

Iodinated casein that was formed under the same conditions that were employed to prepare the product of the highest apparent thyroxine content in this series was used for the isolation of thyroxine (3). Metabolic assays on guinea pigs of the active acid-insoluble products obtained in the course of the isolation procedure indicated that the iodinated casein contained approximately 2 per cent thyroxine.

With this in mind it seemed that the thyroxine content found in the present instance was considerably too high. It seemed possible that the excessive values might be due to incomplete hydrolysis, resulting in split-products that were soluble in *n*-butanol but that still retained some iodinated but non-thyroxine groups in peptide combination with the thyroxine

*Hydrolysis in 40 Per Cent Barium Hydroxide*—In order to effect more extensive hydrolysis the same preparations that were used in the preliminary investigations were hydrolyzed for 20 hours with 40 per cent barium hydroxide. The hydrolysates were then extracted with *n*-butanol, and the iodine content of the extracts was determined as before.

In comparison with the original method (Fig. 1) the results obtained following the more intensive hydrolysis are considerably lower throughout the

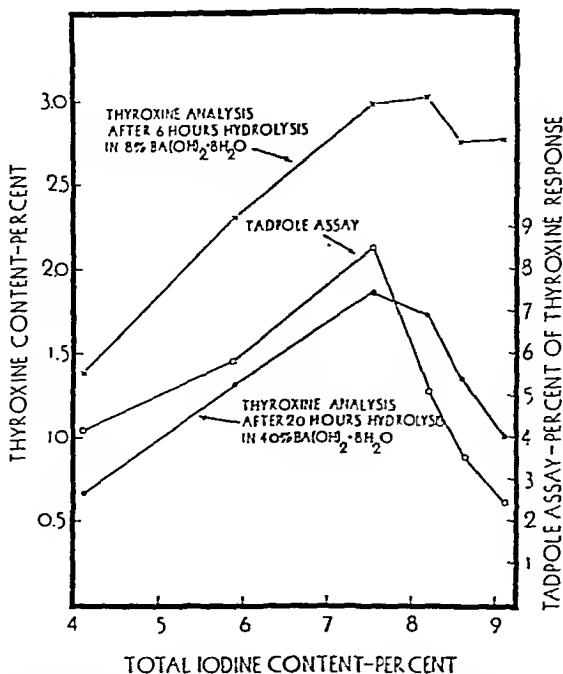


FIG. 1. Apparent thyroxine content of iodinated casein after hydrolysis in 8 and 40 per cent barium hydroxide solution, compared with tadpole assay.

range of iodination. They also show a decline in thyroxine content when the point of optimum iodination is exceeded that is in far better agreement with the biological assay, although still somewhat higher in this region than would be expected. Guinea pig assays check satisfactorily with the chemical procedure over the entire range of progressive iodination (Table IV). The apparent discrepancy could be explained by postulating the formation, with excessive iodination, of linkages in the protein which the tadpole is

unable to metabolize. The value of 1.85 per cent thyroxine obtained for the product of greatest biological potency (Fig. 1) is about what would be expected in a preparation of this type from the previous fractionation data (3).

*Time Required for Hydrolysis in 40 Per Cent Barium Hydroxide*—Iodinated casein samples taken from a preparation that yielded an apparent thyroxine value of 3.4 per cent when hydrolyzed as in Blau's method were digested with 40 per cent barium hydroxide for periods of 4 to 28 hours and analyzed for apparent thyroxine content. The value of 3.11 per cent thyroxine, obtained after hydrolysis for 4 hours, declined to 2.62 per cent in 8 hours and thereafter did not change appreciably during the period up to 28 hours (Table I).

TABLE I

*Effect of Various Periods of Hydrolysis in Barium Hydroxide Solution on Thyroxine Value Obtained on Iodinated Casein Preparation*

Time hydrolyzed	Thyroxine	In Ba(OH) <sub>2</sub> solution
hrs.	per cent	per cent
4	3.11	
6	2.77	40
8	2.62	40
16	2.63	40
20	2.70	40
28	2.58	40
6	3.43	8

The *n*-butanol-soluble fraction obtained after 8 or more hours of hydrolysis is very stable, as is shown by its ability to withstand long continued heating under the conditions employed. If all of the iodine in this portion is calculated as thyroxine, excellent agreement is found between the results thus obtained and the metabolic assay of either the fraction itself or the original iodinated casein, as will be shown later.

In view of the foregoing a 20 hour hydrolysis in 40 per cent barium hydroxide was used in the subsequent analyses.

#### *Analytical Procedure*

*Hydrolysis*—Exactly 1.0 gm. of iodinated casein, 3.2 gm. of Ba(OH)<sub>2</sub>·8H<sub>2</sub>O, and 6.4 ml. of distilled water are placed in a 25 × 200 mm. test-tube. An air-cooled reflux condenser is attached, and the test-tube is placed in a vigorously boiling water bath. When the barium hydroxide has dissolved, the contents of the tube are stirred well, care being taken to work any material adhering to the side walls down into the solution. The prepara-

tion is then replaced in the boiling water bath, and the heating is continued for a total of 20 hours.

The hydrolysate is mixed with 25 ml. of distilled water, and set aside for a few minutes to allow the coarse precipitate of barium salts to settle. The supernatant fluid is then decanted into a 100 ml. volumetric flask. The precipitate of barium salts remaining in the test-tube is decomposed by adding 2 ml. of *n*-butanol and exactly 5.0 ml. of 3.5 *N* hydrochloric acid, shaking, and warming slightly in a water bath to effect complete solution of the precipitate. The dissolved substance is transferred quantitatively to the volumetric flask, and distilled water is added to bring the volume up to 100 ml.

*Extraction*—A 10 or 20 ml. aliquot of the well mixed hydrolysate, containing 2 to 4 mg. of thyroxine, is delivered into a separatory funnel, and 3 drops of 0.5 per cent bromocresol green indicator solution are added. The solution is then titrated with 3.5 *N* hydrochloric acid to a definitely yellow color.

The thyroxine is extracted from the hydrolysate by shaking with 20 ml. of *n*-butanol. The butanol extract is purified in turn by shaking with an equal volume, and then with half the volume of 4 *N* sodium hydroxide solution, containing 5 per cent sodium bicarbonate, as recommended by Blau (8). After each extraction the solutions should be left for an hour or longer to insure good separation before the aqueous layer is drawn off.

The purified *n*-butanol extract is filtered directly into a 100 ml. nickel crucible through a softly packed plug of glass wool that is placed in the stem of the separatory funnel. Finally the separatory funnel is rinsed with 5 ml. of *n*-butanol, which is drained through the glass wool into the crucible. The *n*-butanol is most conveniently removed by evaporation on a steam bath at atmospheric pressure, under a forced draft hood.

The iodine content of the residue is determined by the method of Kendall as described by Harington (10). The per cent thyroxine in the sample is then estimated by multiplying the per cent iodine by 1.529.

In principle the extraction is patterned closely after Blau's method. A smaller sample of iodinated casein is required for analysis than in the case of thyroid substance, however, and the volume of *n*-butanol used for the extraction has been reduced sufficiently so that it can be handled easily in a 100 ml. crucible. This permits evaporation of the solvent and alkaline fusion of the residue in the same container, thus avoiding the manipulations involved in vacuum distillation of the butyl alcohol. In practice we have found that a dozen determinations can easily be carried through at the same time by one operator.

*Recovery of Thyroxine by Extraction Procedure*—Solutions of thyroxine alone and thyroxine plus diiodotyrosine in the amounts to be found in



iodinated casein samples containing 1.5 to 3.0 per cent thyroxine were extracted by the procedure described. Additional samples, with casein added, were carried through the entire hydrolysis and extraction procedures.

TABLE II  
*Recovery of Thyroxine Iodine by Extraction Procedure*

Amount of iodine taken		Found by analysis	
As thyroxine	As diiodotyrosine	Iodine	Calculated as thyroxine
Extracted directly without hydrolysis			
mg.	mg.	mg.	per cent recovery
1.94		1.91	98.3
1.94		1.91	98.3
1.94	9.27	1.95	100.5
1.94	9.27	1.95	100.5
3.88		3.78	97.3
3.88	9.27	3.79	97.6
3.88	9.27	3.82	98.4
	9.27	3.83	98.7
	9.27	0.09	
		0.05	
Average recovery, thyroxine alone.....			97.9
"	"	" with diiodotyrosine.....	99.5
20 hrs. hydrolysis in 40% Ba(OH) <sub>2</sub> ·8H <sub>2</sub> O, with 200 mg. casein added			
3.77		3.41	90.5
3.88	9.27	3.56	91.6
1.94		1.83	94.4
1.94	4.64	1.85	95.4
0.97		0.91	94.3
0.97	2.32	0.92	95.1
	9.27	0.13	
Average recovery, thyroxine alone.....			93.0
"	"	" with diiodotyrosine.....	94.0

As determined by Kendall's method, the thyroxine contained 64.7 per cent and the diiodotyrosine 54.0 per cent iodine. The latter figure indicates that the diiodotyrosine used was the dihydrate.

The recovery of thyroxine alone averaged 97.9 per cent (Table II). With added diiodotyrosine an average of 99.5 per cent of the thyroxine taken was accounted for. These recovery figures are very similar to those reported by Blau (7) by use of a larger volume of *n*-butanol.

As might be expected, some loss of thyroxine occurred when the heating

operation with 40 per cent barium hydroxide solution was continued for 20 hours. The recovery of thyroxine alone averaged 93.0 per cent. In the presence of diiodotyrosine a recovery of 94.0 per cent was obtained.

Diiodotyrosine has but a small effect on the thyroxine value obtained, as is shown by the close agreement in the percentage recovery of thyroxine whether determined in the presence or absence of added diiodotyrosine. If the amount of iodine recovered from diiodotyrosine alone is calculated in terms of thyroxine, the error due to diiodotyrosine iodine in the usual analysis of iodinated casein is found to be  $+0.04$  to  $+0.10$  per cent.

As a further check on this point, an iodinated casein preparation was hydrolyzed alone and together with diiodotyrosine, and analyzed by the usual procedure. As is shown in Table III, the added diiodotyrosine did not increase the result of the thyroxine analysis, although when run alone the diiodotyrosine sample showed an apparent thyroxine content of 0.061 per cent.

TABLE III  
*Effect of Added Diiodotyrosine on Thyroxine Analysis of Iodinated Casein*

Sample taken		Thyroxine found
Iodinated casein	Diiodotyrosine iodine	
gm.	mg.	per cent
1.000	0	3.67
1.000	18.8	3.62
0	19.0	0.06

As a check on the effectiveness of the *n*-butanol extraction of thyroxine from the acidified hydrolysate, analyses were carried out by use of a double extraction with *n*-butanol at this point. An increase of less than 1 per cent of the thyroxine present was obtained.

Blau's data (7) on the distribution ratio between thyroxine in *n*-butanol and the alkaline washing solution indicate the possibility of slight losses of thyroxine in extracting the *n*-butanol extract with alkali. Two iodinated casein samples were analyzed for thyroxine by the usual procedure. The combined alkali extracts from each analysis were reextracted with 30 ml. of *n*-butanol and then washed with an equal volume and half the volume of 4 *N* sodium hydroxide containing 5 per cent sodium carbonate. The *n*-butanol extract was analyzed for iodine as usual.

The two samples contained 3.69 and 3.10 per cent thyroxine as determined by the first analysis. 0.14 and 0.12 per cent of additional thyroxine was obtained by reextraction; this would amount to 3.82 and 3.97 per cent of the original analyses. From these results it would appear that a second *n*-butanol extraction might be desirable if extreme accuracy is required.

The loss of thyroxine in the single extraction is partially offset, however, by the diiodotyrosine retained in the *n*-butanol extract (Table II).

With these results as a basis, the thyroxine content of a group of iodinated casein preparations was determined both by the chemical method and by biological assay in guinea pigs in order to establish the correlation between the two measures.

### *Assay Procedure*

Guinea pigs weighing 600 to 1000 gm., kept in an air-conditioned room held at 25.5°, and fed a ration consisting of 60 per cent dehydrated cereal grass, 20 per cent crimped barley, and 20 per cent rolled oats were used for the assay.

Groups of four healthy animals were used for each trial. On the 1st day of the assay period the animals were deprived of food and water for 4 hours. They were then weighed and the daily dosage for each guinea pig was calculated, based on the individual body weights and the chemically determined thyroxine in the preparation. If no racemization was anticipated, an amount of iodinated casein sufficient to supply 5 to 6  $\gamma$  of thyroxine per 100 gm. of body weight was used. The preparation to be assayed was dissolved in a minimum amount of 0.1 *N* sodium hydroxide and was administered once daily by intraperitoneal injection over a period of 4 days. On the 5th day the feed was withheld for 4 hours, the animals were weighed, and the CO<sub>2</sub> output over a 1 hour period was determined gravimetrically by means of a modified Haldane respiration apparatus. The normal metabolic rate was established by running several hundred determinations on normal untreated guinea pigs. Under the conditions employed, the normal CO<sub>2</sub> output can be calculated by the equation,

$$\text{Gm. CO}_2 \text{ per hr.} = (0.001) \text{ body weight} + 0.1$$

The percentage increase in CO<sub>2</sub> output of the injected animals over the normal values, calculated by the formula, was determined.

Standard response curves were established by injecting purified *l*- and *dl*-thyroxine from the same lots reported on previously (3, 5) (Fig. 2). In confirmation of the earlier reports (5, 11), *l*-thyroxine was found to be twice as active as the racemic mixture.

The percentage increase in CO<sub>2</sub> output of the animals injected with the experimental preparations above the normal values as calculated by the formula was determined. This figure was translated into terms of *l*- or *dl*-thyroxine by means of the standard response curve (Fig. 2).

*Correlation between Results of Thyroxine Analysis and Biological Assay*—The results obtained on a group of iodinated casein preparations by the chemical and biological assays are compared in Table IV. Each figure

represents the average response of four guinea pigs. Since the thyroxine in iodinated casein has been shown to occur in the *l* form (5), the values obtained in the biological assays were computed in terms of *l*-thyroxine.

Preparations 1 to 6 represent data on miscellaneous samples, one of which was assayed biologically three different times. The data for Preparations 7 to 15 were obtained on a series of samples that were prepared by using progressively increasing amounts of iodine, and under conditions somewhat different from those used for preparing the series shown in Fig. 1.

No trend with varying degrees of iodination is observed in the per cent difference obtained, indicating a high degree of specificity for the chemical

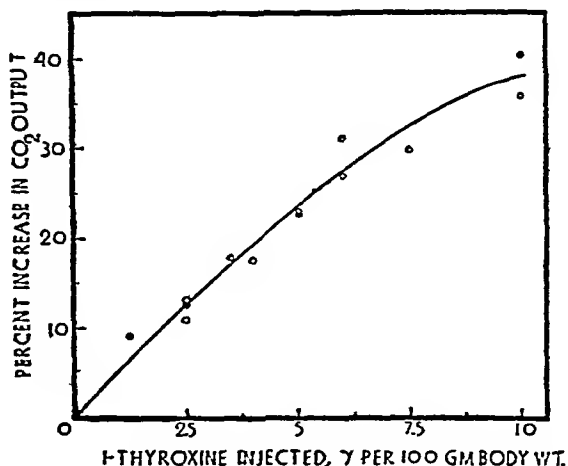


FIG. 2. Standard thyroxine reference curve. The open circles represent points obtained when twice the indicated quantity of *dl*-thyroxine was injected.

method. The fifteen preparations analyzed showed an average thyroxine content of 2.79 per cent determined by biological assay and 3.04 per cent by chemical analysis. The tendency toward slightly lower results by the biological assay may be due to a difference in rate of absorption from the site of injection of the free thyroxine as an assay standard and the thyroxine in the iodinated protein. The possibility remains, however, that non-thyroxine iodine may be causing the slightly higher results obtained with the chemical method.

The data in Table V demonstrate a remarkable difference in absorption, depending upon the site of injection. In the last column the results are calculated as per cent absorption based on the chemical assay value as 100 per cent. Iodinated casein injected intraperitoneally was absorbed to the

extent of almost 95 per cent. When the same preparation was injected subcutaneously, less than half the metabolic response was obtained. In view of the short (5 day) assay period, it is probable that this difference is due to differences in the rate of absorption from the injection sites. It seems likely that the response by the two modes of administration would be equalized over longer periods of administration.

TABLE IV  
*Data Demonstrating Correlation between Chemical and Biological Assay Methods for Thyroxine*

Preparation No.	Iodine added per mole tyrosine in protein	Iodinated protein injected	Increase in CO <sub>2</sub> output	Thyroxine found		
				Bioassay*	Chemical analysis	Difference
	atoms	$\gamma$ per 100 gm. body weight	per cent	per cent	per cent	per cent
1		223	25.4	2.46	2.69	-8.6
2		138	24.7	3.80	3.91	-2.8
3		176	20.8	2.46	3.06	-19.6
4		300	27.6	2.01	2.06	-2.4
5		150	20.9	2.90	3.88	-25.3
6		145	25.1	3.71	3.73	-0.5
6		161	25.0	3.31	3.73	-11.3
6		161	25.1	3.34	3.73	-10.5
7	4.51	243	21.7	1.86	2.21	-15.8
8	5.01	198	23.4	2.50	2.71	-7.7
9	5.51	201	26.8	2.90	2.69	+7.8
10	6.01	190	23.0	2.55	2.83	-9.9
11	6.51	175	23.1	2.78	3.09	-10.0
12	7.01	174	22.2	2.67	3.11	-14.1
13	8.01	191	26.4	2.98	2.83	+5.3
14	9.01	194	23.2	2.53	2.78	-9.0
15	10.01	209	25.8	2.66	2.58	+3.0
Weighted average ...				2.79	3.04	-7.7

\* Estimated from the standard response curve for intraperitoneally injected *L*-thyroxine.

*Metabolic Assay of Iodinated Casein Hydrolysates*—Iodinated casein preparations that had been analyzed for thyroxine were hydrolyzed in 40 per cent barium hydroxide solution by the usual method. The material extracted by *n*-butanol at two different stages in the chemical procedure was assayed by its metabolic stimulation of guinea pigs (Table VI). The results were calculated in terms of *DL*-thyroxine, since hydrolysis in barium hydroxide racemizes the thyroxine (3). The biological assays show excellent agreement with the chemical analyses, indicating that the *n*-butanol

extracts prepared according to the analytical procedure contain all of the thyroxine originally formed in the iodinated protein. Furthermore, all of the thyroidal activity of the original iodinated protein is accounted for by its thyroxine content as determined chemically. While these experi-

TABLE V

*Comparison of Metabolic Effect of Iodinated Casein Administered Intraperitoneally and Subcutaneously*

Method of injection*	Increase in metabolism	Thyroxine equivalent†	Absorption based on chemical assay
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Intraperitoneal	25.0	3.52	94.4
"	25.1	3.54	94.9
Subcutaneous	6.5	0.74	20.0
"	13.0	1.63	43.7‡

\* Iodinated casein containing 3.73 per cent thyroxine by chemical analysis was injected in each case at the rate of 161  $\gamma$  per 100 gm. of body weight daily.

† Based on the standard curve for intraperitoneally injected l-thyroxine.

‡ The metabolism of one animal was high.

TABLE VI

*Data Showing Thyroxine Content of Samples of Iodinated Casein Carried through Different Stages of Chemical Assay Procedure*

	Hydrolysate injected*	Increase in CO <sub>2</sub> output	Thyroxine found		
			Bioassay†	Chemical analysis	Difference
	<i><math>\gamma</math> per 100 gm. body weight</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Acid n-butanol extract	500	24.0	2.04	2.06	-1.0
	150	14.8	3.93	3.88	+1.3
Acid n-butanol extract after washing with alkali	360	23.5	2.76	3.00	-8.0
	412	22.3	2.28	2.62	-13.0
	545	23.9	1.88	1.98	-5.1
	294	24.6	3.57	3.67	-2.7
Average .....			2.74	2.87	-4.75

\* The figures given indicate the amount of original iodinated casein represented.

† Estimated from the standard response curve for intraperitoneally injected dl-thyroxine.

ments do not wholly exclude the occurrence in iodinated proteins of an active compound other than thyroxine, such a compound, if present, would need to have a thyroidal activity per unit of iodine contained in it that is very similar to that of thyroxine iodine, as measured by the guinea pig method.

The method proposed for the quantitative determination of thyroxine in iodinated protein differs from that of Blau (8) for thyroid tissue principally in the use of a more intensive hydrolysis of the starting material. Reduction of the volumes of solution and final elimination of the *n*-butanol solvent at atmospheric pressure also simplify the final steps of the analysis. From the results obtained (Fig. 1 and Table I) it is clear that a vigorous hydrolysis is necessary to bring the material to a state where the thyroxine can be extracted selectively without the inclusion of other iodine compounds. It appears likely that this is due to the linkage of thyroxine with peptide fragments containing other iodinated amino acids which become soluble in *n*-butanol at an early stage in the hydrolysis. By the use of more concentrated barium hydroxide and a longer heating period, thyroxine is liberated as the free compound.

Results in Table II indicate that free thyroxine can be recovered quantitatively by the extraction procedure used. The hydrolysis procedure applied to crystalline thyroxine results in a destruction of 6 to 7 per cent of the thyroxine taken. This high stability is probably due to the fact that thyroxine forms the insoluble barium salt under these conditions and thereafter is very resistant to chemical action.

Comparison of the analytical values with results of the metabolic assays is facilitated by the fact that the iodinated proteins formed by our methods produce their physiological effects when given parenterally without previous hydrolysis (1, 9), and thus can be compared directly with injected thyroxine. By this means differences due to different degrees of absorption from the intestinal tract are avoided, though there is still a small discrepancy, probably due to differences in the absorption of thyroxine and iodinated protein from the injection site (Tables IV and VI).

The data presented also illustrate the high thyroxine content of iodinated casein that can be prepared by present methods. By proper control of the procedure iodinated casein containing 3 to 4 per cent of thyroxine, determined either by chemical analysis or biological assay, can be produced simply and in large quantities. This is 20 to 30 times the thyroxine content of U. S. P. thyroid.

#### SUMMARY

A method is presented for the determination of thyroxine in iodinated casein having thyroidal activity. The method is based upon hydrolysis of the iodinated casein with 40 per cent barium hydroxide solution, extraction of the thyroxine with *n*-butanol, and determination of the iodine content of the purified extract.

It was shown that thyroxine can be recovered quantitatively from known solutions by the extraction procedure. Hydrolysis of thyroxine with 40 per

cent barium hydroxide for 20 hours results in destruction of 6 to 7 per cent of the thyroxine taken. Diiodotyrosine was shown to have a negligible effect on the determination.

Excellent agreement was found between the thyroxine analysis of iodinated casein samples prepared under a variety of conditions and the biological assay value determined by the metabolic stimulation produced by the same preparations when injected intraperitoneally in guinea pigs. *n*-Butanol extracts of iodinated casein prepared exactly as for the chemical method produced a metabolic effect that was fully equivalent to their thyroxine content determined chemically.

From these results it is concluded that any non-thyroxine iodine compounds passing through the chemical procedure with thyroxine either do not comprise more than a small fraction of the total iodine measured, or they must produce a metabolic response in guinea pigs comparable to that produced by thyroxine.

Iodinated casein preparations that can now be formed contain from 3 to 4 per cent of thyroxine, determined by either chemical analysis or biological assay.

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# THE EFFECT OF MANGANESE COMPOUNDS AND CERTAIN OTHER FACTORS ON THE FORMATION OF THYROXINE IN IODINATED CASEIN\*

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In experiments conducted in our laboratory during recent years it has been found that the thyroïdal activity of iodinated proteins is influenced markedly by the conditions employed during their preparation. In earlier communications it has been shown that when the protein is iodinated in a mildly alkaline sodium bicarbonate solution a product of surprisingly high thyroïdal potency is obtained by limiting the addition of iodine to the amount required to substitute 2 atoms per molecule of tyrosine in the protein (1, 2) and, secondly, by incubating the iodinated protein at the elevated temperature of 60–70° (3). It was also observed (3) that the formation of active substance was apparently catalyzed by the metals in a brass stirrer that was used in making up part of the preparations.

From our own experiments (1–3) as well as those of Ludwig and von Mutzenbecher (4), Harington and Pitt Rivers (5), and Johnson and Tewkesbury (6) it was believed that the formation of thyroxine in an iodinated protein must be an oxidative process, involving the coupling of two diiodotyrosine radicals and elimination of one side chain. It thus seemed conceivable that the reaction might be expedited by use of the proper oxidative catalyst and maintenance of mildly oxidative conditions in the reaction medium. Evidence that this is true is provided by the experiments to be reported.

## EXPERIMENTAL

Iodine was combined with the protein in an aqueous medium buffered with sodium bicarbonate by the same general procedure described previously (1). Except in one group of preparations that was maintained at 37° throughout, the iodination was conducted at 38–40°, followed by incubation at 70° for 18 to 20 hours. Factors such as the kind and amount of metallic compound added, the stirring speed, and the amount of iodine and bicarbonate used were varied, as will be indicated in presenting the results.

The thyroxine content of the iodinated casein formed under the various

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conditions was determined by chemical analysis as described by Reineke *et al.* (7).

*Effect of Metallic Compounds and Agitation on Thyroxine Formation*—The previous results (3) suggested that certain metals or metallic compounds might act as catalysts for the formation of thyroxine in iodinated protein. With this in mind samples of iodinated casein were prepared in the presence of copper, copper sulfate, ceric sulfate, cerous sulfate, ferric oxide, and ferric sulfate, but they all contained either the same or a diminished amount of thyroxine compared to the control preparations. When a "colloidal manganese dioxide" solution prepared by the reduction of  $\text{KMnO}_4$  with glucose (8) was added, however, there was a consistent increase in the thyroxine content of the resulting iodinated casein. It seemed likely that the solution used would contain a mixture of the various oxides of manganese. Therefore, the effect on the reaction of a number of oxides and also of manganese sulfate was tested.

In all of the recent experiments on the formation of iodinated casein having thyroidal activity, the reaction mixture has been stirred vigorously throughout the procedure, although evidence that this is essential has not been reported. It seemed of interest, therefore, to determine what effect the degree of agitation might have on the thyroxine content of the resulting product.

One group of preparations (Table I) was formed by iodinating the proteins of skim milk with minimum stirring throughout the process. Sodium bicarbonate was added at the rate of 5 gm. per 700 ml. of milk, and then powdered iodine was added slowly in an amount sufficient to provide 4.5 atoms per mole of tyrosine in the protein (1). The temperature was maintained at  $37^\circ$  during the iodination and subsequent holding period of 18 to 20 hours. For the remaining samples listed in Table I, 20 gm. of casein were suspended in 700 ml. of distilled water containing 5 gm. of sodium bicarbonate, and 3.7 gm. of powdered iodine were added by the usual procedure at  $38\text{--}40^\circ$ . Small amounts of various manganese compounds were added to part of the preparations, either before or after the addition of iodine. The reaction mixtures were then incubated at  $70^\circ$ , with stirring as indicated in Table I, for 18 to 20 hours.

It will be noted that at  $37^\circ$  and with very gentle stirring a product containing less than 0.3 per cent of thyroxine was formed. By increasing the incubation temperature to  $70^\circ$  and stirring vigorously, but without any added catalyst, the thyroxine formation was increased to an average of 1.76 per cent in five preparations.

The addition of 20 mg. of  $\text{Mn}_2\text{O}_4$  caused only a slight increase to 1.96 per cent thyroxine when the stirring motors were adjusted to only 300 R.P.M. However, the thyroxine content of the iodinated protein increased to an

average of 2.88 per cent when  $Mn_2O_3$  was added and stirring was maintained at 600 R.P.M. The amount of  $Mn_2O_3$  added, within the range of 10 to 40 mg. per sample, appears to have no effect on the result.

TABLE I

*Effect of Incubation Temperature, Manganese Compounds, and Amount of Agitation on Formation of Thyroxine in Iodinated Protein*

Catalyst	Stirring	Thyroxine content	Average
Series I. Skim milk proteins iodinated and incubated at 37°			
None	r.p.m. Very gentle	per cent 0.33	
"	" "	0.26	
"	" "	0.27	0.29
Series II. Casein iodinated at 38-40°, incubated at 70°			
None	300	1.67	
"	600	1.73	
"	600	1.80	
"	600	1.75	
"	600	1.84	1.76
$Mn_2O_3$	300	1.94	
"	300	1.99	1.95
"	600	2.72	
"	600	2.93	
"	600	3.03	
"	600	2.78	
"	600	2.80	
"	600	3.04	2.88
Oxides from reduction of $KMnO_4$	600	2.97	
"	600	2.96	
"	600	2.60	2.84
$MnO_2$	600	2.16	
"	600	2.19	2.17
$Mn_2O_3$	600	2.26	
"	600	2.33	2.30
$MnSO_4$	600	2.00	
"	600	2.13	2.07

Results practically identical to those obtained by the addition of  $Mn_2O_3$  were observed by use of the oxides formed by the reduction of  $KMnO_4$ . Small, but consistent, increases in thyroxine content over that of the control preparations were obtained by the addition of  $MnO_2$ ,  $Mn_2O_3$ , and  $MnSO_4$ . The latter compounds thus appear to be less effective than the first two in promoting the formation of thyroxine, but it seems possible

that this may be explained by failure to use the most effective concentration of these substances.

The fact that  $\text{MnSO}_4$  as well as the various oxides of manganese caused some increase in thyroxine formation indicates that the manganese ion itself is the important factor in catalyzing the reaction. Increased thyroxine formation was observed whether the manganese compound was added before the iodination or immediately preceding the incubation period.

*Effect of Manganese Oxide and Increasing Bicarbonate and Iodine Concentration on Thyroxine Formation in Iodinated Casein*—Earlier experi-

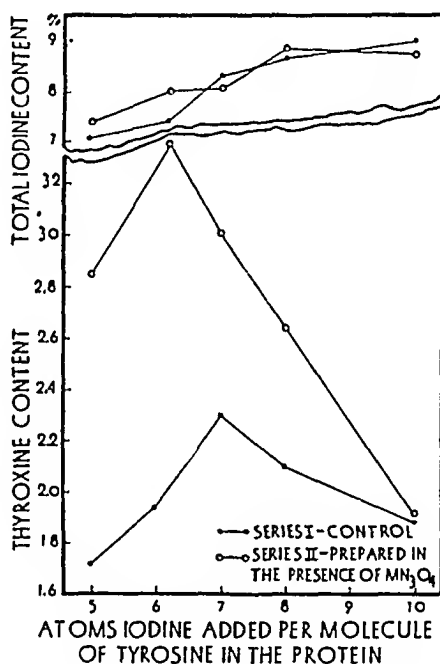


FIG. 1. The effect of progressive iodination in the presence of excess bicarbonate and  $\text{Mn}_2\text{O}_4$  on the thyroxine content of iodinated casein.

ments (1, 2, 7) indicated the critical nature of the amount of iodine used in limiting the amount of thyroxine formed under the prescribed conditions. In the initial report (1) it was observed that when the iodine input was increased progressively in preparations buffered with a constant amount of sodium bicarbonate the final pH of the solutions declined in samples that were iodinated excessively, owing to depletion of the buffer capacity of the solutions by hydriodic acid that is formed as a side product in the reaction. Thus, it seemed possible that this decline in pH, if carried below the optimum level for the formation of thyroxine, might become a limiting factor.

As a further check on this point, two series of iodinated casein preparations were made up by the procedure employed previously (2) except that the sodium bicarbonate added was increased proportionately with the iodine in successive preparations. In all cases, sodium bicarbonate equivalent to 1.35 times the weight of the iodine to be used was added before iodination was begun. Under these conditions the pH of all of the solutions was maintained within the range of approximately 8.0 to 8.5 throughout the process. No catalyst was added to the control preparations (Series I). In Series II, 20 mg. of  $Mn_3O_4$  were added to each preparation before iodination was begun. The stirring motors were adjusted to 600 R.P.M. in all samples.

Under the previous conditions (1, 2) maximum thyroïdal activity was obtained when 4.5 to 5 atoms of iodine were added per mole of tyrosine in the protein. With the reaction adjusted as described, however, thyroxine formation continued to increase (Fig. 1) until slightly more than 7 atoms of iodine had been added. In the presence of  $Mn_3O_4$  (Series II), maximum thyroxine formation occurred with the addition of 6 atoms of iodine per mole of tyrosine. By addition of the catalyst, thyroxine formation at the optimum iodination level was increased from 2.28 to 3.37 per cent. With excessive iodination there was a significant decline in thyroxine formation, but to a smaller extent than was observed under the conditions used formerly. The  $Mn_3O_4$  had no significant effect on the total iodine content of the preparations, as is shown in the upper part of Fig. 1.

#### DISCUSSION

The bulk of the evidence now available indicates that the formation of thyroxine in a protein is brought about first by iodination of the tyrosine. Under the proper conditions 2 molecules of the diiodotyrosine thus formed are oxidatively coupled with the elimination of one side chain to form thyroxine. Assuming the tyrosine content of casein to be 5.65 per cent (1), the theoretical yield of thyroxine would be 10.6 per cent. The maximum thyroxine content of 3.37 per cent observed in the data reported (Fig. 1) constitutes slightly more than 30 per cent of the theoretical conversion. In instances in which proper attention has been given to all of the factors now known to influence this reaction, iodinated casein containing 4 per cent or more of thyroxine has been prepared (7).

The mechanism first proposed by Johnson and Tewkesbury (6) and elaborated further by Harington (9) provides a plausible theoretical background for the formation of thyroxine by oxidative coupling of the diiodotyrosine formed during iodination of the protein. It has been necessary to determine the optimum conditions for the reaction by experimentation, however. It was suggested (6, 9) that the coupling reaction is brought

about by the oxidative action of iodine. The fact that some extra iodine above that required for substitution of 2 atoms per mole of tyrosine is required for maximum thyroxine formation is in line with this idea. The amount of excess iodine that can be used appears to depend on the pH of the medium. Under the conditions used formerly (1-3) the iodine was limited to approximately 4 to 5 atoms per mole of tyrosine. In solutions containing a greater excess of bicarbonate more iodine can be added before more extensive action, resulting in losses of activity, takes place.

The fact that greatly increased thyroxine formation occurs in preparations incubated at 60-70° (3) after iodination at 38-40° suggests that during the iodination the principal reaction is that of substituting iodine in tyrosine and that the coupling reaction occurs in large part during the incubation step.

Kohler and Hoover<sup>1</sup> have found that the formation of thyroxine in iodinated casein is diminished markedly if the iodination and incubation processes are carried out under nitrogen. When the iodination only was conducted under nitrogen and the reaction mixture was then aerated vigorously during the incubation period, a large increase in the thyroxine content of the iodinated casein was observed. The increase in thyroxine formation with vigorous agitation, as noted in the present report (Table I), is believed to be brought about by the oxidative action of the atmospheric oxygen that is whipped continuously into the reaction mixture. This view is verified by further experiments by the authors on the direct formation of thyroxine from diiodotyrosine (unpublished). If diiodotyrosine is incubated under conditions similar to those established for iodinated casein, only traces of thyroxine are formed if the solution is left undisturbed. Large increases in thyroxine formation are brought about by stirring the solutions vigorously in contact with air, or by bubbling air through them.

Manganese will accelerate the reaction only in the presence of oxygen. It thus appears to act as an oxygen carrier for the oxidative coupling reaction that is involved.

The rôle of manganese in catalyzing the formation of thyroxine in iodinated proteins *in vitro* is of special interest biologically in view of the finding of Ray and Deysach (10) that the thyroid has a special capacity for the storage of manganese and, further, that the injection of traces of this compound causes an increase in the oxygen consumption of guinea pigs.

This together with the data reported in the present paper suggests that manganese may act catalytically *in vivo* as well as *in vitro* in promoting the formation of thyroxine in the thyroid gland.

<sup>1</sup> Kohler, G. O., and Hoover, R. D., Cerophyl Laboratories, Inc., Kansas City, Missouri, personal communication.

## SUMMARY

The effect of a number of factors on the formation of thyroxine in iodinated casein, as determined by chemical analysis, is reported.

It is shown that, subsequent to the iodination of the protein, the amount of thyroxine formed is increased markedly by increasing the incubation temperature to 70° and agitating the solution very vigorously. The principal effect of agitation is believed to be the incorporation of atmospheric oxygen, which then brings about oxidative coupling of 2 molecules of diiodotyrosine within the protein to form thyroxine.

The formation of thyroxine is shown to be catalyzed by a series of Mn compounds, including  $\text{MnSO}_4$ ,  $\text{Mn}_2\text{O}_4$ ,  $\text{Mn}_2\text{O}_3$ ,  $\text{MnO}_2$ , and the oxides of Mn obtained by the reduction of  $\text{KMnO}_4$  by glucose. Best results were obtained with the latter compounds and with  $\text{Mn}_2\text{O}_4$ .

From these results, together with the reported capacity of the thyroid for storing manganese, it is suggested that manganese may act catalytically *in vivo* as well as *in vitro* in promoting the oxidative formation of thyroxine in the thyroid gland.

It is also shown that, by the use of a larger excess of sodium bicarbonate than was employed in the earlier experiments, a larger amount of iodine can be added to the protein before a decline in thyroxine formation takes place. The thyroxine formation at the point of optimum iodination is greater than that observed under the former conditions.

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# A DIRECT METHOD FOR DETERMINATION OF OXYGEN AND CARBON DIOXIDE TENSIONS IN BLOOD\*

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Although satisfactory techniques for the determination of oxygen and carbon dioxide contents of blood have long been available, methods for the determination of gas tensions have been less satisfactory and, in the case of the most frequently used procedures, indirect ((1) pp. 174, 298). The direct methods ((1) p. 309; (2-7)) depend upon the equilibration of a bubble of gas with blood at 37° and analysis of the bubble for carbon dioxide and oxygen.

In the method to be described the technical problems inherent in the direct approach are minimized by the use of an instrument recently devised by Roughton and Scholander for micro gasometric analyses (8). The Roughton-Scholander (R-S) syringe, consisting of a syringe to which a calibrated capillary tube has been fused, is used both as equilibration chamber and bubble analyzer.

## EXPERIMENTAL

The accuracy of the bubble technique was determined by comparing tensions thus obtained with those obtained by a tonometer technique. Blood was equilibrated at 37° with a large volume of gas in a tonometer, and, after drawing off the blood the tensions of carbon dioxide and oxygen in the gas in the tonometer were determined by analysis in the Haldane apparatus. Simultaneously the tensions of carbon dioxide and oxygen in the blood withdrawn from the tonometer were determined by the bubble method. The gas tensions in the tonometer were considered the standard of accuracy and any discrepancy between these results and those of the bubble analysis was considered to indicate inaccuracy in the bubble method.

The accuracy of determinations of tension on arterial blood drawn at altitude and analyzed at sea-level was estimated by experiments performed

\* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

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in the low pressure chamber at altitudes between 7700 and 14,200 feet. Alveolar air samples were taken before and during the blood sampling and were analyzed immediately for oxygen by an almost instantaneous physical method (tensimeter). The oxygen tension of the chamber air was likewise determined to give an accurate estimate of equivalent altitude. Gas tensions and oxyhemoglobin saturations of the blood drawn at altitude were determined at sea-level. With these data it was possible to compare the determined values for alveolar oxygen tension and arterial oxygen tension under conditions of moderate anoxia. It was also possible to compare both the arterial carbon dioxide and the arterial oxygen tensions with the average alveolar tensions found by Helmholtz *et al.* (9) at the corresponding equivalent altitude. Furthermore, with determined values for both oxygen tension and oxygen saturation of the blood, it was possible to plot a point on the oxygen dissociation curve. In this preliminary work the pH was not determined, but a rough comparison was made with standard dissociation curves (10). Together these comparisons provided a reasonable basis for evaluating the accuracy of the blood gas tension technique as applied to blood drawn at altitude.

The rate of change in oxygen and carbon dioxide tensions in freshly drawn arterial blood containing 0.01 to 0.02 per cent sodium fluoride was measured by performing determinations at specified intervals.

### *Bubble Method for Determination of Blood Gas Tension*

#### *Materials and Reagents—*

1. Two Roughton-Scholander syringes (30 unit or, preferably, 50 unit capillary).
2. Heparin solution (Lederle), 20 per cent, and sodium fluoride, 4 per cent, in physiological saline solution. 1 drop for each 4 ml. of blood provides a concentration of sodium fluoride in blood of 0.05 per cent.
3. Needle with a rubber adapter (described in the text).
4. Toothpick swabs (cotton).
5. Alveolar air bag (a rubber bag from a sphygmomanometer cuff, with a pinch-clamp on one outlet tube and a hypodermic needle in the other).
6. Water bath (37°) with a rotating device.
7. Interval timer.
8. Syringes, one of 10 ml. capacity for the blood sample, three of 5 ml., with 21 gage needles, for the saline solution, the sodium hydroxide solution, and the alkaline hydrosulfite solution, and two of 2 ml., with hypodermic needles, for the mercury and "waste."
9. Stock solution of sodium chloride, 0.9 per cent.
10. Stock solution of sodium hydroxide, 4.0 per cent.

11. Alkaline hydrosulfite solution ( $\text{Na}_2\text{S}_2\text{O}_4$ ). Make up fresh daily as follows: To 0.5 gm. of sodium hydrosulfite add 2.5 ml. of 5.6 per cent (1.0 N) KOH solution. Stir briefly, filter immediately through cotton, and draw into a 5 ml. syringe. Expel the air bubbles.

### *Procedure*

*Preparation of R-S Syringe*—The R-S syringe is cleaned by rinsing first with water, then with 0.1 N lactic acid, and finally with physiological saline solution.<sup>1</sup> The metal clip on the syringe is so adjusted that a definite though slight resistance is offered to motion of the plunger.

*Elimination of Air from Dead Space in Syringe and Capillary*—About 0.1 ml. of mercury and a little saline solution are drawn into the barrel of the syringe. The syringe is held in the upright position so that the mercury rests on top of the plunger and saline solution fills in around the mercury meniscus. The plunger is completely advanced, pushing out all air bubbles and leaving the capillary and cup filled with mercury and a trace of saline solution. Saline solution and mercury are withdrawn from the top until the cup is left about half full of mercury.

*Sampling of Blood Anaerobically*—1 drop of heparin-fluoride solution for each 4 ml. of blood is introduced into a sampling syringe and a few small drops of mercury are added to aid in mixing. The dead space in the syringe is eliminated and the desired amount of arterial or venous blood introduced with care to avoid sucking in air bubbles. The syringe is immediately rotated.

*Filling R-S Syringe*—A needle with a rubber adapter permitting a snug fit into the cup of the R-S syringe<sup>2</sup> is attached to the syringe containing the blood sample. A little blood is introduced into the cup of the R-S syringe so that it forms a layer on top of the mercury and fills the cup without trapping air bubbles. The needle is then advanced into the cup until the rubber adapter makes a tight seal. Both syringes are now held in the vertical position with the sampling syringe inverted and the R-S syringe upright. By pressing on the plunger of the sampling syringe, the R-S syringe is filled to the 1 ml. mark under pressure. The R-S syringe should always be examined to make sure that no air bubbles are present.

<sup>1</sup> For the determinations reported in this paper a phosphate buffer solution (pH 7.3) was used in place of physiological saline. There is no necessity for this refinement in technique provided syringes are carefully cleaned and kept uncontaminated with acid or alkaline reagents.

<sup>2</sup> Such an adapter can be made by pushing a No. 23 needle through a solid piece of rubber stopper until the rubber abuts against the base of the needle. The size of the piece of rubber should be so chosen that the tip of the needle projects about 1 cm. The rubber can be whittled down with scissors until it has a conical taper permitting a snug fit into the cup of the R-S syringe.

*Admission of Alveolar Air Bubble*—A small rubber bag, such as the bag in the inflatable cuff of a sphygmomanometer, is used for storing alveolar air. One of the two rubber outlet tubes is fitted over the base of a hypodermic needle, and the other is closed with a pinch-clamp. The operator fills the bag in advance with his own alveolar air, so that he can liberate a fine jet through the needle as needed by squeezing the bag.

The cup of the R-S syringe is emptied with the "waste" syringe. Care is taken to prevent the formation of small bubbles in the blood at the junction of the capillary and the cup of the R-S syringe. The tip of the alveolar air needle is placed near the bottom of the cup and the bag squeezed, thus flushing the cup with alveolar air. Simultaneously the plunger of the R-S syringe is withdrawn until the desired amount of alveolar air has been introduced into the capillary (25 units for an expected  $pO_2$  of 60 mm. of Hg or higher, 35 units for an expected  $pO_2$  of 40 to 60 mm. of Hg, 45 units for an expected  $pO_2$  of less than 40 mm. of Hg).<sup>3</sup> The cup is then half filled with blood from the sampling syringe, and, with the R-S syringe in the upright position, the measured bubble is drawn into the barrel of the syringe. The syringe is then turned to the horizontal position so that the bubble can float freely away from the capillary.<sup>4</sup> The capillary is left full of blood but the cup is left at least partially empty, so that air will separate the blood from the water of the 37° bath.

*Equilibration*—The syringe now contains 1 ml. of blood, an alveolar air bubble, and a little mercury. Equilibration is accomplished by rotating the R-S syringe in a 37° water bath for 7 minutes. To insure good mixing it is desirable to have lengthwise as well as rotary motion of the mercury and the bubble. This can be accomplished by rotating the syringe on a truncated conical block.

*Ejection of Mercury and Most of Blood*—The R-S syringe is removed from the rotating device and instantly inserted into a 100 ml. cylinder containing water at 37°.<sup>5</sup> Since the syringe is inverted, the mercury and most of the blood can be expelled into the cylinder without losing the bubble. When about 0.1 ml. of blood remains, the syringe is momentarily removed from the water bath and turned to the upright position. The bubble is slowly and smoothly advanced into the capillary during the next few seconds. Half the blood in the cup is then quickly sucked out and the syringe is replaced in the water bath, cup end first. In this way,

<sup>3</sup> These specific quantities apply only when the capillary bore is 0.5 mm. See the discussion and Table IV.

<sup>4</sup> When this technique is properly performed, there is no tendency for the bubble to break up and there is therefore no need to use caprylic alcohol to reduce surface tension. Caprylic alcohol is, in fact, contraindicated, because it interferes with the drainage of blood from the capillary wall in the subsequent bubble analysis.

<sup>5</sup> This cylinder is submerged in the 37° water bath.

air is trapped in the cup, and maintains a separation between blood and water. The syringe is laid horizontally on a tray about 2 inches below the surface of the water.

*Bubble Analysis*—The volume of the bubble,  $V_1$ , is read against a white background with the syringe held in the horizontal position and just below the surface of the water in the 37° bath. The bubble length is estimated to 0.1 unit.

The syringe is then taken out of the water. All gross blood is removed from the cup with the "waste" syringe and the cup is filled with saline solution. This solution is drawn into the capillary as far as the 50 unit mark,<sup>6</sup> thus diluting and washing down the blood in the capillary. The remaining saline solution in the cup is removed and the cup filled with 4.0 per cent sodium hydroxide solution. With the R-S syringe in the upright position the alkali is slowly drawn down until its upper meniscus enters the capillary and falls to the 50 unit mark. The blood in the barrel of the syringe now forms a lower layer and the bubble floats above in a clear layer of alkali solution. By advancing the plunger of the syringe slowly, the analyst now pushes the bubble back into the capillary with care to obtain good drainage. The syringe is replaced on the tray in the 37° water bath, and, after a brief period for temperature equalization, the second reading is taken.  $V_2$  represents the volume of the bubble after  $\text{CO}_2$  absorption.

The R-S syringe is again removed from the water bath and the cup is sucked empty with the "waste" syringe. The cup is then filled with alkaline hydrosulfite solution, and this is slowly drawn down with the R-S syringe in the upright position, until the upper meniscus of the hydrosulfite enters the capillary and reaches the 50 unit mark. The bubble is slowly pushed back into the capillary, the syringe is replaced in the 37° water bath, and  $V_3$ , representing bubble volume after  $\text{O}_2$  absorption, is read.

*Calculations*—

$$\% \text{CO}_2 = \frac{V_1 - V_2}{V_1}$$

$$\text{Mm. CO}_2 = \% \text{CO}_2 \times (\text{barometric pressure} - 47)$$

$$\% \text{O}_2 = \frac{V_2 - V_3}{V_1}$$

$$\text{Mm. O}_2 = \% \text{O}_2 \times (\text{barometric pressure} - 47)$$

Empirical correction factors, based on a comparison of results by the bubble method with those by the tonometer method, may be used to give increased accuracy.

<sup>6</sup> For 30 unit syringes read the 30 unit mark.

In this laboratory the results tabulated in Table I have led to the use of +2 mm. as the correction factor for  $p\text{CO}_2$  and -3 mm. as the factor for  $p\text{O}_2$ . Individuals learning the technique should if possible establish their own correction factors on the basis of tonometric standards.

*Technique of Performing Simultaneous Duplicate Analyses*—Duplicate analyses can be performed simultaneously by one person if one R-S syringe is manipulated while the other rests in the water bath. Both syringes are rotated together for the 7 minute equilibration but the various stages of the subsequent bubble analysis are performed alternately on one syringe and then the other. The time required is but slightly greater than for a single analysis.

In all instances simultaneous duplicate analyses are performed, and checks of 0.8 per cent (6 mm. of Hg) or better are required for both oxygen and carbon dioxide.

### *Tonometer Technique*

*Tonometer Equilibration*—Tonometers were prepared by introducing a gas mixture with tensions in the physiological range. 5 ml. of blood were then introduced and the tonometer rotated in a water bath at 37° for at least 20 minutes. Atmospheric pressure was maintained by opening the stop-cock momentarily after 5 minutes and again after 10 minutes.

*Removal of Blood from Tonometer*—In preparation for the withdrawal of blood from the tonometer the needle with the tapered rubber adapter described above was attached to a 10 ml. syringe, and all air was displaced from the syringe and needle with mercury and saline solution. At the end of the equilibration period the tonometer was removed from the rotating device and held in the vertical position in the water bath until drainage was virtually complete. It was then removed from the water and the needle immediately pushed into the bore of the glass tubing at the lower end of the tonometer until the rubber adapter made a tight seal. The tonometer stop-cock was first opened to the side arm, so that the dead space could be filled with mercury, and then opened to the body of the tonometer. The blood was withdrawn, and the gas in the tonometer was immediately put under positive pressure by introducing mercury from a leveling bulb.

*Analysis of Gas in Tonometer*—Standard techniques for sampling and analyzing gases in the Haldane apparatus were used. The percentages of carbon dioxide and oxygen in the gas in the tonometer were determined in duplicate, and checks of 0.08 per cent or better were required.

*Determination by Bubble Method of Gas Tensions in Blood from Tonometer*—Immediately after withdrawal from the tonometer, the blood was introduced into a pair of R-S syringes, and carbon dioxide and oxygen tensions were determined by the bubble technique described above.

## Results

Twenty-three comparisons were made between determinations of tensions by the bubble method and by the tonometer method. On one occasion duplicate bubble analyses failed to check each other. The results of

TABLE I

*Comparison of Bubble Method with Tonometer Method in Determination of Gas Tensions in Identical Blood Samples*

Experi- ment No.	Carbon dioxide					Oxygen				
	Bubble			Tono- meter	Differ- ence	Bubble			Tono- meter	Differ- ence
	per cent	Aver- age per cent	mm. Hg			per cent	Average per cent	mm. Hg		
1	5.6, 5.2	5.4	39	42	-3	14.9, 14.7	14.8	106	106	0
2	6.6, 6.6	6.6	47	49	-2	11.9, 11.9	11.9	85	78	+7
3	5.3, 5.9	5.6	40	43	-3	13.7, 13.7	13.7	98	95	+3
4	4.6, 5.0	4.8	34	35	-1	5.4, 5.3	5.35	38	36	+2
5	3.5, 4.1	3.8	27	27	0	4.9, 5.0	4.95	35	32	+3
6	5.6, 5.5	5.55	40	41	-1	6.1, 6.0	6.05	43	38	+5
7	3.9, 3.9	3.9	28	32	-4	15.3, 15.6	15.45	111	111	0
8	6.6, 6.2	6.4	46	50	-4	10.0, 10.3	10.15	73	71	+2
9	4.6, 4.3	4.45	32	36	-4	8.4, 8.9	8.65	62	51	+11*
10	3.5, 3.4	3.45	25	33	-8	8.2, 8.0	8.1	58	47	+11*
11	6.4, 6.4	6.4	46	48	-2	12.6, 12.8	12.7	91	88	+3
12	4.6, 4.2	4.4	32	34	-2	6.6, 7.0	6.8	49	47	+2
13	3.6, 3.9	3.75	27	28	-1	8.5, 8.7	8.6	62	53	+9*
14	3.8, 4.6	4.2	30	34	-4	9.6, 9.2	9.4	67	61	+6
15	8.3, 8.5	8.4	60	50	+10*	3.6, 3.7	3.65	26	24	+2
16	4.1, 4.9	4.5	32	33	-1	8.5, 9.1	8.8	63	55	+8
17	5.6, 5.7	5.65	41	45	-4	13.0, 12.5	12.75	92	87	+5
18	4.9, 5.2	5.05	36	40	-4	8.6, 8.8	8.7	62	52	+10*
19	6.2, 6.0	6.1	44	42	+2	8.1, 7.3	7.7	55	52	+3
20	4.9, 4.6	4.75	34	34	0	7.4, 7.7	7.55	54	51	+3
21	5.7, 6.0	5.85	42	43	-1	3.2, 3.3	3.25	23	22	+1
22	5.7, 5.7	5.7	41	43	-2	12.5, 12.8	12.65	91	87	+4
Average.....					-2.3					+3.3

\* Not used in calculation of the average differences.

the remaining twenty-two consecutive experiments are summarized in Table I and Figs. 1 and 2.

One carbon dioxide determination was grossly in error, probably due to a unit error in reading the capillary calibrations. This determination has been omitted in calculating the average differences between results of the two methods. Four oxygen determinations were omitted in cal-



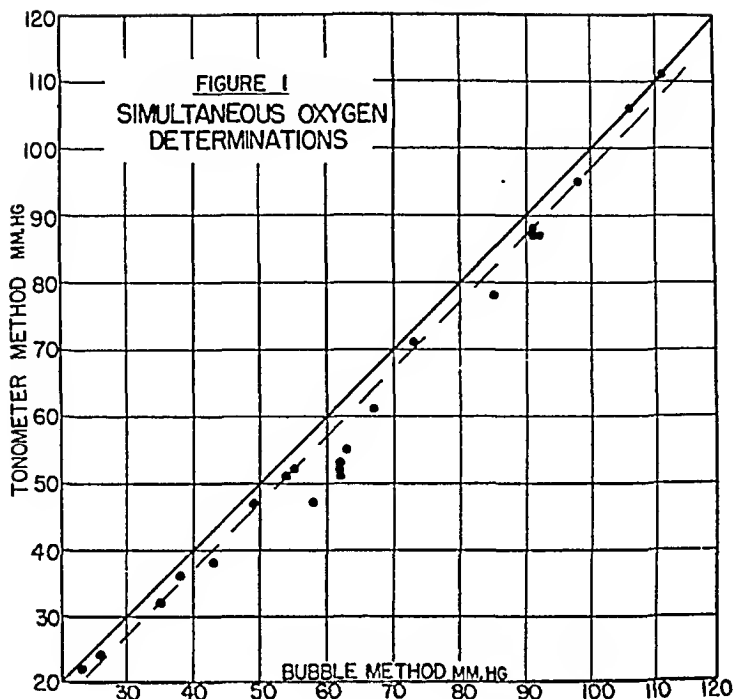


Fig. 1

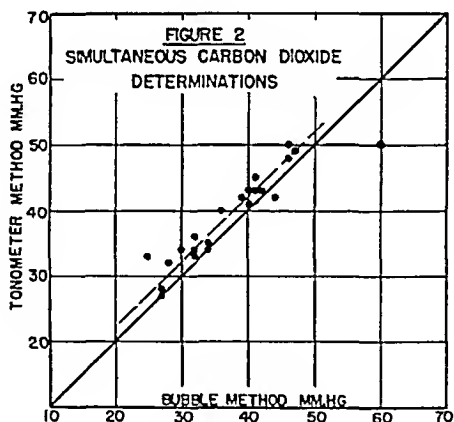


Fig. 2

culating the average differences. All these four were in the 45 to 55 mm. range by Haldane analysis, and the errors probably resulted from the use of too large bubbles during equilibration.<sup>7</sup>

<sup>7</sup> Accurate results have since been obtained in this tension range with bubbles chosen strictly in accord with Table IV.

In most instances the method is accurate to  $\pm 3$  mm. of Hg for both carbon dioxide and oxygen.

In Table II arterial oxygen tensions are compared to alveolar oxygen tensions under conditions of moderate anoxia (a simulated altitude of 7700 to 14,200 feet). The subjects were not adequately trained in giving alveolar samples, and there was a considerable spread in alveolar oxygen tensions as determined. The oxygen tensions in alveolar air and arterial blood fell in the same range, however. There was also a satisfactory correspondence between the arterial carbon dioxide and oxygen tensions and the average alveolar tensions found by Boothby at equivalent altitudes.

TABLE II

*Gas Tensions in Arterial Blood Drawn at Altitude (Low Pressure Chamber); Correlation with Alveolar Air and Oxyhemoglobin Saturation*

Subject	Low pressure chamber, $pO_2$	Equivalent altitude	$O_2$ tension				$CO_2$ tension		$HbO_2$ saturation
			Alveolar			Arterial	Alveolar, Boothby*	Arterial	
			Boothby*	Tensimeter†					
	mm. Hg	ft.	mm. Hg	mm. Hg		mm. Hg	mm. Hg	mm. Hg	per cent
Gem	117	7,700	67	61(+3, -3) 68 65	Before During After	59	36	33	
Fra	110	9,300	61	57(+3, -5) 56	Before During	67	36	31	87
Hil	95	13,000	48	47(+8, -3) 45	Before During	49	35	34	85
Bea	95	13,000	48	45(+7, -1) 59	Before During	44	35	30	76
Pro	90	14,200	45	48(+6, -4) 45	Before During	51	34	28	79

\* From Helmbolz, Bateman, and Boothby (9).

† Average tensions obtained before, during, and after sampling of the arterial blood.

In no instance was the discrepancy beyond the range of variation among normal subjects. In the four instances in which the arterial oxyhemoglobin saturation was determined, points were plotted on the oxygen dissociation curve. Two of the four points fell close to the pH 7.4 curve. The other two points were somewhat to the right of the standard curve, possibly due to erroneously high values for arterial oxygen tension.<sup>2</sup>

In Table III the rate of change in carbon dioxide and oxygen tensions in shed blood containing 0.01 to 0.02 per cent sodium fluoride is shown

<sup>2</sup> The bubbles used were too large, and oxygen transfer may have raised the tension of the blood by as much as 3 or 4 mm. of Hg.

for twelve carefully timed experiments. Carbon dioxide tensions usually increased after  $\frac{1}{2}$  hour, probably because the blood was treated with an

TABLE III

*Rate of Change in Oxygen and Carbon Dioxide Tensions in Shed Blood Containing 0.01 to 0.02 Per Cent Sodium Fluoride*

Subject	Time*	Temperature	pCO <sub>2</sub>	pO <sub>2</sub>
	min.	°C.	mm. Hg	mm. Hg
Ril	3	24	37	110
	35	24	42	104
	65	24	42	99
Pro	4	24	32	103
	35	24	36	95
Lil†	3	24	37	103
	35	24	40	97
"†	10	24	38	102
	20	24	38	74
	30	24	43	67
	40	24	38	98
Fug	12	24	38	98
	60	0	38	91
	240	0	40	88
Lil	5	24	35	87
	35	0	37	80
Pau	5	24	23	82
	40	0	26	79
Din	5	24	36	61
	35	24	38	52
Lil	5	24	50	19
	35	0	49	21
"	5	24	55	19
	35	0	56	21
Ril	5	24	47	21
	35	0	49	23
Lil	5	24	47	51
	35	24	45	56

\* The time in minutes refers to the interval between the sampling of the blood and the beginning of the bubble equilibration.

† The samples were taken during carbon monoxide experiments. The blood contained 3.6 volumes per cent of carbon monoxide. The extremely rapid rate of decline in oxygen tension in the second sample was unique and remains unexplained.

insufficient quantity of sodium fluoride.<sup>9</sup> Oxygen tensions in the higher ranges dropped several mm. after  $\frac{1}{2}$  hour at room temperature, and this

<sup>9</sup> Subsequent work has shown that, when the concentration of sodium fluoride in blood is 0.05 per cent, the carbon dioxide tensions remain virtually constant.

tendency persisted when the blood was chilled. In the lower ranges of oxygen tension the rate of change was much slower.

#### DISCUSSION

*Bubble Characteristics*—If the oxygen tension of blood is to be determined by allowing the sample to equilibrate with a bubble of air, the transfer of gases which takes place in the process of attaining equilibrium must cause no significant alteration in the oxygen tension of the sample. Inevitably some slight alteration will occur, and, for any given oxygen tension, there is a definite and predictable amount of oxygen which can be added to or taken from a 1 ml. blood sample without causing the oxygen tension of the sample to change by more than 1 mm. of Hg. If this amount is first estimated, it is then possible to define the characteristics of a bubble which, in coming to equilibrium, will neither add to nor take away from the blood more than this amount.

From a standard oxygen dissociation curve it can be seen that in the 20 mm. of Hg tension range a change in tension of 1 mm. of Hg involves a 2.5 per cent change in saturation; in the 60 mm. of Hg range it involves a 0.4 per cent change; and in the 100 mm. of Hg range it involves only a 0.1 per cent change. The addition of 0.2 c.mm. of oxygen to 1 ml. of blood whose oxygen capacity is 20 volumes per cent will raise the oxygen saturation by 0.1 per cent. In coming to equilibrium with blood at 100 mm., therefore, the bubble must exchange not over 0.2 c.mm. of oxygen with the blood.

If the analyst collects an alveolar air sample whose oxygen tension is  $100 \pm 20$  mm. of Hg and if the oxygen tension of the blood is 100 mm. of Hg, then the tension of the bubble at the start of equilibration will not differ from that of the blood by more than 20 mm. During equilibration the oxygen tension of the bubble will change by a maximum of 20 mm., corresponding to an oxygen transfer of  $20/713$  or 2.8 per cent of the total bubble volume. Therefore, 2.8 per cent of the bubble volume must not exceed 0.2 c.mm., and the total bubble volume must not exceed 7.1 c.mm. This, then, is the largest bubble which can be used to equilibrate with a 1 ml. sample of blood in the 100 mm. of Hg oxygen tension range, when the oxygen tension of the bubble at the start of equilibration is within 20 mm. of that of blood, without causing an alteration in the blood oxygen tension of more than 1 mm. of Hg.

7.1 c.mm. correspond to 18 R-S capillary units when the capillary bore is 0.5 mm. (1 unit = 2 mm.). A bubble this size provides a bubble to blood volume ratio of 1:141.

Bubble characteristics for blood oxygen tensions throughout the entire physiological range are presented in Table IV. The choice of bubble

size to use in any given blood oxygen tension range varies with the capillary bore of the R-S syringe. It is undesirable to use bubbles which are less than 25 units long, because the reading errors with such small bubbles become more significant than the errors due to oxygen transfer when a 25 unit bubble is used. Since 50 unit capillaries are the longest available at present, it is not possible to use bubbles which are larger than this even though, as indicated in Table IV, there would be no theoretical objections in the lower tension ranges.

*Time Required for Equilibration*—The time required for gaseous equilibria to reach completion depends upon the tension gradient between gas and

TABLE IV  
*Bubble Characteristics*

The maximum change in oxygen tension of a 1 ml. blood sample = 1 mm. of Hg. The oxygen capacity of blood was assumed to be 20 volumes per cent; the oxygen tension of the alveolar air bubble, 120 mm. of Hg at the start of equilibration.

Blood			Bubble					Ratio of bubble volume to blood volume
$pO_2$	$O_2$ saturation change for 1 mm. Hg tension change	$O_2$ required for 1 mm. Hg tension change	$O_2$ transferred	Bubble volume				
				Capillary bore*				
				0.4 mm.	0.5 mm.	0.6 mm.		
<i>mm. Hg</i>	<i>per cent</i>	<i>c.mm.</i>	<i>per cent of bubble volume</i>	<i>c.mm.</i>	<i>R-S units</i>	<i>R-S units</i>	<i>R-S units</i>	
100	0.1	0.2	2.8	7.1	28	18	11	1:141
80	0.2	0.4	5.6	7.1	28	18	11	1:141
60	0.4	0.8	8.4	9.5	38	24	14	1:105
50	0.7	1.4	9.8	14.3	57	36	22	1:70
40	1.3	2.6	11.2	23.2	93	58	35	1:43
20	2.5	5.0	14.0	35.7	143	89	54	1:28

\* For a 0.4 mm. bore, 100 R-S units (Roughton-Scholander syringe) equal 25 c.mm.; for a 0.5 mm. bore, 40 c.mm.; for a 0.6 mm. bore, 66 c.mm.

blood, upon the specific characteristics of the gas, and upon whether or not a chemical combination takes place tending to speed the equilibrium to completion. We have found empirically that a 7 minute equilibration period is adequate for carbon dioxide and oxygen.

*Nitrogen Tensions*—The gas which contributes the largest proportion of the total tension in blood, as in air, is nitrogen. While the nitrogen tension of an alveolar air bubble is essentially the same as that of arterial blood, it is higher than that of venous blood and much higher than that of blood drawn at altitude. When a considerable difference in nitrogen tensions exists between the bubble and blood at the start of a determination, nitrogen equilibration may not reach completion in 7 minutes. (Serial measurements show that the bubble is still shrinking.) After 5 or 6

minutes, however, the rate at which nitrogen moves from the bubble to the blood is so slow that carbon dioxide and oxygen equilibria are not significantly disturbed.

#### SUMMARY

1. A direct method for the determination of carbon dioxide and oxygen tensions in blood is described.

2. The method depends upon the equilibration of a bubble of gas with blood at 37° and the analysis of the bubble for carbon dioxide and oxygen.

3. The Roughton-Scholander syringe (preferably with a 50 unit capillary) is used both as the equilibration chamber and bubble analyzer, thus eliminating the necessity for transfer of the bubble.

4. At sea-level, the accuracy of the technique was tested by comparison of results with those of a tonometer technique. The results agreed within 3 mm. of Hg in nineteen out of twenty-two cases for carbon dioxide and in sixteen out of twenty-two cases for oxygen.

5. At simulated altitudes of 7700 to 14,200 feet (low pressure chamber), the accuracy of the technique was evaluated by comparing arterial tensions with alveolar tensions. The agreement between simultaneously determined arterial and alveolar oxygen tensions was good. The agreement was also good between determined oxygen and carbon dioxide tensions in arterial blood and average alveolar tensions found by Helmholtz and coworkers at equivalent altitudes.

6. The carbon dioxide tension of shed blood containing 0.01 to 0.02 per cent sodium fluoride usually increased after  $\frac{1}{2}$  hour, probably because the blood was treated with an insufficient quantity of sodium fluoride. The oxygen tension of arterial blood dropped several mm. after  $\frac{1}{2}$  hour at room temperature, and this tendency persisted when the blood was chilled. The oxygen tension of chilled venous blood remained comparatively constant.

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# THE PROTEOLYTIC INHIBITING SUBSTANCE IN THE EXTRACT FROM UNHEATED SOY BEAN MEAL AND ITS EFFECT UPON GROWTH IN CHICKS\*

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The nutritional significance of enzymatic inhibitors which modify the action of enzymes acting in the digestive tract has not been heretofore studied, although the possibilities of such significance have been mentioned in some of the recent publications upon naturally occurring inhibitors (1-3). It has been suggested that they may be of importance in bacterial nutrition also (4, 5).

It has been known for many years that the growth-promoting value of soy beans is greatly enhanced by heating (6). This increase in nutritional value of the heated soy beans was attributed by Hayward and Hafner (7) and by Almquist, Mecchi, Kratzer, and Grau (8) to the general raising of the level of the sulfur-bearing amino acids (cystine and methionine) by heat treatment. They have pointed out, however, that this amino acid deficiency may not have been the only limiting growth factor eliminated by autoclaving. It was also found by Almquist *et al.* that supplemental methionine resulted in a stimulation of growth when fed with heated soy bean meal.

The explanation that heat enhances the value of proteins is inconsistent with the generally accepted belief that elevated temperatures tend to lower the nutritional value of most proteins (9-11). In addition, Thayer and Mussehl<sup>1</sup> demonstrated that a growth-inhibiting property of raw soy beans was primarily due to a substance present in the raw soy beans which could be extracted with water, leaving a residue which compared favorably with autoclaved meal in nutritional value. It was likewise shown by them that the addition of methionine and cystine increased the nutritional value of both heated and unheated soy bean meal.

Because of the similarity of properties of the growth-retarding factor extractable from unheated soy beans and the trypsin-inhibiting substance also found in these extracts, it appeared highly probable that the two were identical (3).

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† With the technical assistance of Clinton Koppes.

<sup>1</sup> Thayer, R. H., and Mussehl, F. E., unpublished data from the Nebraska Department of Poultry Husbandry.



Studies were designed, therefore, to demonstrate whether partially purified solutions of the proteolytic inhibitor would produce lowering of the rate of growth. Experiments were also designed to demonstrate the inhibition of trypsin *in vitro* by the material found in the small intestines of chicks which had been fed a ration composed of uncooked soy bean meal.

*Preparation of Inhibitor for Feeding Trials*—Oil-free soy bean flake, prepared with a minimum of heat treatment, was used as the material for extraction.<sup>2</sup> When heated soy bean meal was required, it was prepared by autoclaving the raw flake for 45 minutes at 15 pounds pressure. The flake was finely ground before extraction.

3000 gm. of the ground, unheated soy bean flake were added to 15 liters of water containing sufficient acid (114 ml. of concentrated hydrochloric acid) to bring the final mixture to pH 4.2, which is the isoelectric point of most of the soy bean proteins (12). Extraction was carried out overnight in the cold (4°), after which the mixture was filtered through cloth and the filtrate centrifuged with a Sharples supercentrifuge at 40,000 r.p.m. Only about 5 to 7 per cent of the original soy bean protein remained in the final extract. The residue from the first extraction was reextracted to minimize loss of the soluble inhibitor.

*Precipitation of Inhibitor from Extracts*—The proteolytic inhibiting substance could be precipitated from the extracts, along with protein, by adding enough acetone to make a 70 per cent solution. The inhibiting substance was dissolved from this precipitate with water. Further purification was accomplished by reprecipitation from 70 per cent acetone. The material thus obtained was easily dissolved (or peptized) in water. The concentration of inhibitor in the second precipitate was from 8 to 10 times that found in the raw soy bean flake. Solutions of this second acetone precipitate were used in both feeding trials.

### Procedure

*Feeding Trials*—For each trial, thirty-two 2 week-old single comb white Leghorn chicks of uniform size and vigor were selected and placed in individual heated batteries. The chicks were divided into two groups of sixteen birds. Each chick was fed and weighed individually and a feed consumption record was kept for each bird. The feeding methods described by Ackerson, Blish, and Mussehl (13) were employed. Weights were taken at about 3 day intervals during the trials. The feeding experiments were continued for 15 days.

In Trial I the basal ration consisted of a mixed feed in which 50 per cent of the protein was furnished by autoclaved soy bean meal (23 per cent of the ration). In Trial II all of the protein supplement was of animal origin;

<sup>2</sup> Prepared under the direction of J. W. Hayward by the Archer-Daniels-Midland Company, Minneapolis, Minnesota.

i.e., meat scraps, fish meal, and dried buttermilk. The composition of the rations is given in Table I.

The inhibitor was dissolved in water and the required aliquot carefully placed in the crop of each chick by means of a fast flowing pipette. The solutions were administered to the chicks three times daily at the time of each feeding. In Trial I the control chicks were given water instead of the inhibitor. In Trial II the controls were given a solution of the autoclaved inhibitor. *Efficiency of gain* as used in this paper is the total gain divided by the weight of the feed consumed.

TABLE I  
*Composition of Basal Rations Used in Feeding Trials*

	Trial I	Trial II
	<i>per cent</i>	<i>per cent</i>
Yellow corn meal	35 0	36 0
Bran	10 0	20 0
Pulverized oats	10 0	10 0
Alfalfa meal (dehydrated)	5 0	5 0
Autoclaved soy bean meal	23 0	
Fermentation by-product	3 0	
Meat scraps		5 0
Fish meal		5 0
Dried buttermilk		5 0
Calcium carbonate	1 8	1 8
Sodium chloride (iodized)	1 0	1 0
Manganese sulfate	0 2	0 2
Irradiated sterol (D blend, 200 units vitamin D per gm )	1 0	1 0
	100 0	100 0
Total protein of ration	21 3	20.0

To determine the effectiveness of the inhibiting substance on the trypsin of the chick intestinal tract the following procedure was used. Four 4 week-old chicks (250 to 300 gm. in weight) which had been raised on a well balanced ration were given *ad libitum* only raw soy bean meal for a period of 4 days, whereas four others were given only autoclaved meal during the same period. The chicks were then killed and the contents of that portion of the intestines starting at a point just above the duct of the pancreas and ending at the juncture with the ceca were removed by washing. The suspension of intestinal contents thus obtained from each chick was diluted to a 50 ml. volume and an aliquot of 5 ml. used as the enzyme for digestion of 50 ml. of a 5 per cent casein substrate at pH 7.5. Proteolysis was determined by formol titration.

A 70 per cent acetone precipitate was also prepared from the water extract of the intestinal contents of these chicks and its inhibitory effect determined upon a digest consisting of 4 per cent trypsin (Armour's 1:75) acting on a 5 per cent casein substrate at pH 7.5.

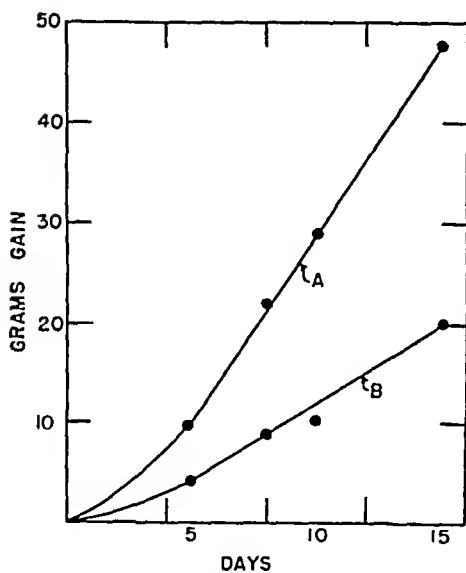


FIG. 1

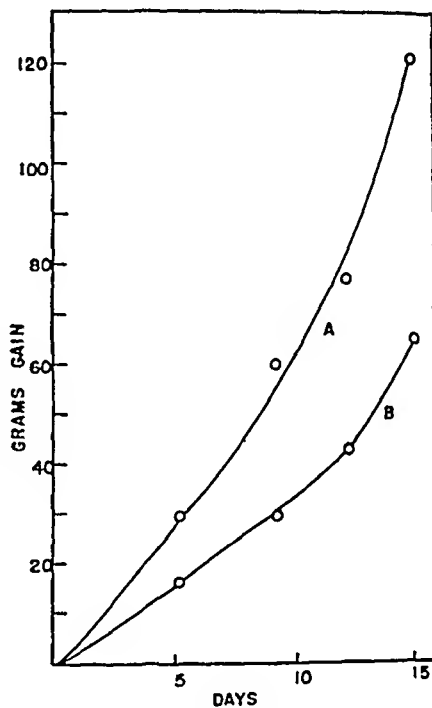


FIG. 2

FIG. 1. Effect of feeding the proteolytic inhibitor from unheated soy bean meal on the growth rate of chicks fed a basal ration containing 23 per cent autoclaved soy bean meal. Curve A, received no inhibitor; Curve B, received 0.3 gm. of acetone-precipitated inhibitor three times daily.

FIG. 2. Effect of feeding the proteolytic inhibitor from raw soy bean meal on the growth rate of chicks fed a basal ration containing supplemental protein of animal origin. Curve A, received 0.3 gm. of autoclaved acetone-precipitated inhibitor three times daily; Curve B, received 0.3 gm. of unheated acetone-precipitated inhibitor three times daily.

### Results

*Feeding Trials*—Figs. 1 and 2 show that chicks receiving the solution of prepared inhibitor exhibited a decreased gain compared to chicks receiving water or a solution of the inhibitor which had been inactivated by autoclaving and administered in the same manner. The depression of growth

was found in the chicks receiving either the basal ration containing supplemental protein composed entirely of autoclaved soy bean meal (Fig. 1) or

TABLE II  
Average Gain and Efficiency of Gain for Groups of Chicks in Feeding Trials

Trial No.	Group No.	Average total gain	Total feed consumed	Total protein	Gain per gm. feed	Gain per gm. protein
		gm.	gm.	gm.	gm.	gm.
I	1. Fed proteolytic inhibitor	20.5	141	29.61	0.145	0.69
	2. Positive controls (water)	47.1	164	34.44	0.287	1.37
II	1. Fed proteolytic inhibitor	71.0	256	51.20	0.277	1.39
	2. Positive controls (solution of inactivated inhibitor)	112.0	306	61.20	0.366	1.83

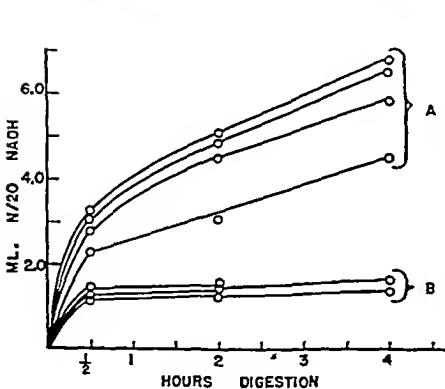


FIG. 3

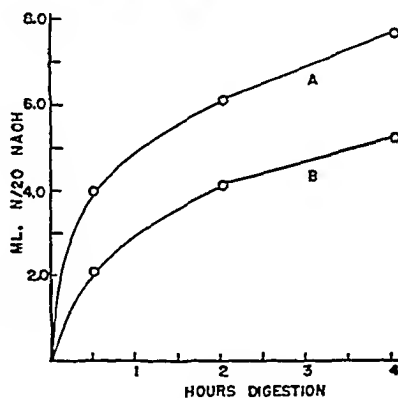


FIG. 4

FIG. 3. Effect of feeding autoclaved *versus* raw soy bean meal on the proteolysis produced by the material obtained from the small intestine of chicks. The action of material from the intestines of four birds which were fed (Curve A) a diet of autoclaved soy bean meal, or (Curve B) a diet of raw soy bean meal.

FIG. 4. Inhibition of proteolysis by means of the inhibitor precipitated from the intestinal contents of chicks which had been fed raw soy bean meal. Comparison of tryptic digestion in the presence of precipitates obtained from the intestinal contents of chicks fed (Curve A) autoclaved soy bean meal or (Curve B) unheated soy bean meal.

the one with no soy bean meal but supplemented by proteins from animal sources (Fig. 2). There was a difference, both in total gain and in the

efficiency of gain between the controls on the two rations, the ration containing the high proportion of autoclaved soy bean protein possessing a lower growth-promoting value. On the high soy bean diet, the chicks given the solution of inhibitor showed an efficiency of gain of 0.145 gm. as compared with 0.287 gm. for the control chicks (Table II). In the case of the ration containing supplemental protein of animal origin, the efficiency of gain of chicks receiving an equivalent amount of inhibitor was 0.277 gm. as compared to 0.366 gm. for the controls.

*Effectiveness of Inhibitor upon Proteolysis in Vitro*—That the proteolytic inhibitor had a pronounced effect on the proteolysis occurring in the chick intestinal tract is indicated by the data presented in Fig. 3. The curves represent the proteolysis produced by the intestinal contents from individual birds, four of which had been fed a diet of raw soy bean meal and four a diet of autoclaved soy bean meal for a period of 4 days. It is evident that the proteolytic activity of the intestinal contents of the chicks was lowered by the diet of unheated soy bean meal.

*Precipitation of Proteolytic Inhibitor from Intestinal Contents*—The proteolytic inhibiting substance could be precipitated by 70 per cent acetone from extracts of the intestinal contents taken from the chicks receiving the diet of uncooked soy bean meal. As shown in Fig. 4, solutions of this acetone precipitate decreased the rate of proteolysis produced by trypsin.

## DISCUSSION

The effect of inhibiting the digestion of protein and the consequent limitation upon growth would be expected to be dependent upon the level of protein fed. If the quantity of inhibitor present is below that required for maximum inhibition, the quantity should determine the efficiency of protein digestion and utilization. Inasmuch as the total proteolytic digest is only retarded, with a constant amount of inhibitor present the level of protein fed should also influence the amount of protein digested and accordingly should affect the growth rate.

It will be noted from the data presented in Figs. 1 and 2 that there was considerable difference in the rate of gain of the chicks in the two feeding trials. The growth of the chicks receiving the diet containing the high level of soy bean protein, even when autoclaved, was far below the growth obtained with the diet containing protein known to be adequate. Some of this difference may have been due to palatability, as shown by the feed consumption data (Table II). However, large differences were also shown in the efficiency of gain of the chicks in the two trials.

When a high proportion of soy bean meal was fed (50 per cent of total protein), chicks given the solution of inhibitor showed a rate of gain which was only 50 per cent of that for the controls. Chicks given an equivalent

amount of the inhibitor but receiving a protein supplement composed of more complete proteins made a rate of gain which was 70 per cent of that of the controls. This difference indicates that the type and quality of the protein fed modified the effect of the proteolytic inhibitor upon growth and, conversely, that the effects of deficiencies in the proteins of the diet may be intensified by the presence of proteolytic inhibitors.

Since the amino acid level and composition of a protein, in itself, may be a limiting factor as to its nutritional adequacy and therefore its growth-promoting value, in the case of a protein which contains a slight suboptimal level of one or more essential amino acids, the available level of the amino acids may be decreased by inhibition of proteolysis to the point where they become definite amino acid deficiencies; in fact, the first amino acid or acids which fell below requirement levels because of the lack of digestion would become the limiting factors upon growth. By this reasoning, methionine, which is suboptimal even in heated soy bean protein (6) (in which the proteolytic inhibitor has been inactivated) should appear as a limiting growth factor in any comparison between heated and unheated soy beans. It is probable that the growth response which supplemental methionine gives with heated soy bean meal represents the true growth stimulation due to methionine enrichment of the soy bean protein, while the much greater effect of supplemental methionine with the unheated meal is due both to the actual suboptimal quantity of the amino acid and to the decreased availability of that which is present in the meal.

The demonstration that enzyme inhibitors occurring in natural food-stuffs may cause deficiencies in rations which in themselves are adequate opens another field in nutritional research. It is obvious that substances which prevent complete digestion, either of proteins or of carbohydrates, may cause deficiencies even though the diet may be nutritionally complete. Enzymatic inhibitors similar to the trypsin inhibitor found in soy beans and the amylase inhibitors found in some of the cereals are probably present in other natural products. Nutritionally, others of these may be of considerable importance. The need for considering this possibility in studying the efficiency of rations is indicated.

#### SUMMARY

1. The proteolytic inhibiting substance present in unheated soy bean meal had a retarding effect upon the growth of chicks when given with either a ration containing autoclaved soy bean meal or one containing a supplement composed of nutritionally adequate proteins from animal sources.

2. The proteolytic inhibitor had a greater effect on growth when fed with a ration supplemented with soy bean protein than when fed with a diet supplemented with more complete animal proteins.

3. When the contents of the small intestines of chicks receiving a diet of raw soy bean meal were used as the enzyme on a substrate of 5 per cent casein (pH 7.5), proteolytic action was greatly retarded, compared to the action of the intestinal contents from chicks receiving a diet of autoclaved soy bean meal.

4. It was possible to precipitate the proteolytic inhibiting substance from water extracts of the intestinal contents of chicks that had been fed uncooked soy bean meal.

5. An explanation is given for the apparent greater methionine deficiency of raw soy bean meal as compared to autoclaved meal.

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# INVESTIGATIONS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

## XXIII. THE DETERMINATION OF PHENYLALANINE IN PROTEIN HYDROLYSATES WITH *LEUCONOSTOC MESAENTEROIDES* P-60 AND *LACTOBACILLUS CASEI*\*

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During the past 2 years microbiological procedures<sup>1</sup> have been reported for the determination in proteins of thirteen of the twenty-one amino acids which occur in protein materials. Seven amino acids (isoleucine, glutamic acid, methionine, leucine, phenylalanine, tryptophane, and valine) have been determined with *Lactobacillus arabinosus* 17-5, three (arginine, tyrosine, and valine) with *Lactobacillus casei*, two (histidine and lysine) with *Leuconostoc mesenteroides* P-60, two (aspartic acid and serine) with *Lactobacillus delbrückii* (4), and one (tryptophane) with *Eberthella typhosa* T-63 (5). Comparable methods (unpublished) have been developed in the authors' laboratory for the determination of cystine, glycine, and threonine. It is possible, therefore, to determine microbiologically all of the accepted amino acids except alanine, proline, hydroxyproline, 3,5-diiodotyrosine, and thyroxine.

It has been shown, previously, that phenylalanine<sup>2</sup> is essential for the growth of *Clostridium sporogenes* (7), *Corynebacterium diphtheriae* (8), *Streptococcus hemolyticus* (9), *Lactobacillus manniopoeus* (10), *Lactobacillus buchneri* (10), *Lactobacillus lycopersici* (10), *Lactobacillus delbrückii* (11), *Lactobacillus arabinosus* 17-5 (12-16), *Lactobacillus casei* (3, 17), and *Leuconostoc mesenteroides* P-60 (18). Although Hegsted (15) has assayed phenylalanine with *Lactobacillus arabinosus* 17-5, the present studies were undertaken

\* For Paper XXII in this series see Camien *et al.* (1). This work was aided by grants from the Gelatin Products Company, Merck and Company, Inc., the Nutrition Foundation, Inc., Schering and Glatz, and the University of California. The authors are indebted to H. Block, W. Frankl, B. Merrifield, and L. B. Rockland for valuable suggestions and technical assistance.

<sup>1</sup> Literature references have been given by Dunn *et al.* (2). According to Baumgarten *et al.* (3) cystine, glutamic acid, isoleucine, leucine, threonine, tryptophane, and valine can be determined with *Lactobacillus arabinosus* and arginine, glutamic acid, isoleucine, leucine, phenylalanine, serine, tryptophane, tyrosine, and valine with *Lactobacillus casei*.

<sup>2</sup> It is of interest that Kidder and Dewey (6) have shown recently that phenylalanine is essential for the growth of the ciliated protozoan, *Tetrahymena geleii* W.



because it appeared that the values reported for phenylalanine and other amino acids in casein and other proteins were less accurate than those obtained for the amino acids determined by analogous published methods. The determination of phenylalanine in protein hydrolysates with *Lactobacillus casei* and *Leuconostoc mesenteroides* P-60 is described in the present paper.

#### EXPERIMENTAL

The assay technique described in previous publications from this laboratory was employed in the present experiments. Separate solutions of basal media, amino acid test mixtures, protein hydrolysates, standard amino acid, sodium chloride, and inocula were delivered to 4 inch test-tubes with the aid of an automatic pipette (Baltimore Biological Company instrument). The total volume in each case was 3 ml. All of the solutions were adjusted to the same concentration of sodium chloride to compensate for any stimulatory or inhibitory salt effects. The standard (phenylalanine) was run at eight levels (0 to 16  $\gamma$ ), the amino acid test mixtures and protein hydrolysates were run at five levels, and from five to six tubes were employed at each level of sample and from eight to twelve tubes at each level of standard. By using numbers of tubes more nearly statistically significant than those usually employed, intrinsic errors inherent in microbiological procedures were compensated and assay values of relatively high accuracy were obtained. It was found as satisfactory to titrate the mixture of solutions from five or more tubes at a particular level of sample as to titrate the individual solutions and calculate the average titration value.

A basal medium essentially of the composition shown in Paper XVIII (18) (Medium D, Table I, with phenylalanine omitted) was employed for the assay of phenylalanine with *Leuconostoc mesenteroides* P-60. This basal medium was modified to contain 1.3 and 2 times the amounts of the amino acids given in the table, since it was found from experiments on multiple amino acid media that the quantity of acid produced at any given concentration of phenylalanine was nearly constant at both levels of total amino acids.

The basal medium utilized for the determination of phenylalanine with *Lactobacillus casei* was essentially the same as that originally described by Hutchings and Peterson (12) and that employed previously (17) in the authors' laboratory for the assay of phenylalanine and other amino acids in an amino acid test mixture. The composition of this medium modified for use in the present experiments was *dl*-alanine 200 mg., natural asparagine 200 mg., *l*(+)-arginine monohydrochloride 200 mg., *l*(+)-glutamic acid 200 mg., *l*(-)-histidine monohydrochloride monohydrate 200 mg., *dl*-

isoleucine 200 mg., *l*(-)-leucine 200 mg., *l*(+)-lysine monohydrochloride 200 mg., *dl*-methionine 200 mg., *dl*-threonine 200 mg., *l*(-)-tryptophane 200 mg., *l*(-)-tyrosine 200 mg., *dl*-valine 200 mg., *l*(-)-cysteine hydrochloride 275 mg., *dl*-serine 300 mg., and folic acid 6  $\gamma$  per liter of solution. An equivalent amount of cysteine hydrochloride was substituted for cystine

TABLE I  
Results of Assays of Phenylalanine in Amino Acid Test Mixture 1\*

Amino acid mixture per tube	<i>Leuconostoc mesenteroides</i>				<i>Lactobacillus casei</i>			
	Titration volume of 0.02N NaOH per tube		Phenylalanine found†		Titration volume of 0.02N NaOH per tube		Phenylalanine found‡	
	Range†	Average	Per tube	Per ml. sample	Range‡	Average	Per tube	Per ml. sample
$\gamma$	ml.	ml.	$\gamma$	$\gamma$	ml.	ml.	$\gamma$	$\gamma$
84.4	3.40-3.75	3.56	2.36	4.72	3.51-3.70	3.60	2.38	4.76
168.8	5.07-5.46	5.27	4.75	4.75	4.92-5.15	5.03	4.60	4.60
253.2	6.64-7.00	6.81	7.30	4.86	5.95-6.39	6.20	7.00	4.67
337.6	7.82-8.33	8.14	9.50	4.75	6.97-7.19	7.05	9.25	4.62
422.0	9.20-9.74	9.42	12.00	4.80	7.69-7.78	7.74	11.62	4.67

\* The composition of the test mixture simulating casein was the same as that given in Paper XVII (20) except that the mixture contained 2.83 per cent of *l*(-)-phenylalanine (as *dl*-phenylalanine). The basal medium  $\times$  2 and 5 days incubation time at 35° were employed with *Leuconostoc mesenteroides* and 3 days incubation time at 35° was employed with *Lactobacillus casei*.

† Five replicate tubes at each level of sample.

‡ An average of 4.78  $\gamma$  of phenylalanine was found per ml. of amino acid test mixture. The average mean deviation from the mean at the different levels was 0.9 per cent. The percentage of phenylalanine recovered in the assay was 100. The following percentages of phenylalanine were recovered in analogous experiments: 100 and 100 (basal medium  $\times$  1.3, 4 days incubation), 101 and 103 (basal medium  $\times$  1.3, 5 days incubation), 102 and 101 (basal medium  $\times$  2, 4 days incubation), and 100 (basal medium  $\times$  2, 5 days incubation).

§ An average of 4.67  $\gamma$  of phenylalanine was found per ml. of amino acid test mixture. The average mean deviation from the mean at the different levels was 0.9 per cent. The percentage of phenylalanine recovered was 98. The recovery of phenylalanine in an analogous experiment was 105 per cent.

because of the low solubility of the latter amino acid. The amounts of serine and folic acid were increased, because the need for larger quantities of these constituents was shown in unpublished experiments. Ammonium chloride was omitted and the remaining constituents (except amino acids) were the same as those given in Medium D, Table I, of Paper XVIII (18).

It was shown in a series of experiments with *Lactobacillus casei* and *Leuconostoc mesenteroides* P-60 that *dl*-phenylalanine had 50 per cent of

the activity of *l*(-)-phenylalanine. The mean deviations from the theoretical response of *dl*-phenylalanine were  $\pm 2.6$  per cent at fourteen concentrations (0 to 30  $\gamma$ ) with *Leuconostoc mesenteroides* and  $\pm 3.0$  per cent at twelve concentrations (0 to 50  $\gamma$ ) with *Lactobacillus casei*. The

TABLE II

Results of Assays of Phenylalanine in Amino Acid Test Mixture 2\*

Amino acid mixture per tube	<i>Leuconostoc mesenteroides</i>				<i>Lactobacillus casei</i>			
	Titration volume of 0.028 N NaOH per tube		Phenylalanine found†		Titration volume of 0.028 N NaOH per tube		Phenylalanine found‡	
	Range†	Average	Per tube	Per ml. sample	Range†	Average	Per tube	Per ml. sample
$\gamma$	ml.	ml.	$\gamma$	$\gamma$	ml.	ml.	$\gamma$	$\gamma$
98.4	4.28-4.47	4.37	2.47	12.35	4.30-4.50	4.43	2.49	12.45
196.8	6.20-6.48	6.34	4.97	12.42	5.60-5.78	5.67	4.87	12.18
295.2	7.89-8.09	8.01	7.34	12.23	6.40-6.71	6.55	7.20	12.00
393.6	9.30-9.79	9.50	9.79	12.24	6.98-7.34	7.17	9.44	11.80
492.0	10.71-11.09	10.90	12.19	12.19	7.55-8.00	7.79	12.00	12.00

\* Test Mixture 2 contained 100 mg. per 100 ml. of each of the amino acids listed in Test Mixture 1 except that the mixture contained 2.44 per cent of *l*(-)-phenylalanine (as *dl*-phenylalanine). The basal medium  $\times 2$  and 5 days incubation at 35° were employed with *Leuconostoc mesenteroides* and 3 days incubation at 35° was employed with *Lactobacillus casei*.

† Six replicate tubes at each level of sample.

‡ An average of 12.28  $\gamma$  of phenylalanine was found per ml. of amino acid test mixture. The average mean deviation from the mean at the different levels was 0.49 per cent. The recovery of phenylalanine was 102.1 per cent. The recoveries of phenylalanine in analogous experiments with test mixtures containing lower percentages of phenylalanine were 104 per cent (0.99 per cent of phenylalanine, basal medium  $\times 2$  and 5 days incubation time), 105 per cent (0.50 per cent phenylalanine, basal medium  $\times 2$  and 5 days incubation time), and 108 and 112 per cent (0.17 per cent phenylalanine, basal medium  $\times 2$  and 5 days incubation time).

§ An average of 12.08  $\gamma$  of phenylalanine was found per ml. of amino acid test mixture. The average mean deviation from the mean at the different levels was 1.5 per cent. The recovery of phenylalanine was 100.5 per cent. The recoveries of phenylalanine in analogous experiments with test mixtures containing lower percentages of phenylalanine were 102 per cent (0.99 per cent phenylalanine), 106 per cent (0.50 per cent phenylalanine), and 117 and 120 per cent (0.17 per cent phenylalanine).

*l*(-)-phenylalanine employed was prepared by Dr. A. Recsei in this laboratory by resolution of *dl*-phenylalanine according to the procedure of Fischer and Schoeller (19). Stokes and Gunness (16) reported in 1944 that only the natural antipode of phenylalanine is active in promoting growth of *Lactobacillus casei*.

The casein, the silk fibroin, and the hydrolysis procedure were the same as those described earlier (20). The experimental data are given in Tables I to VII.

## DISCUSSION

It has been found that phenylalanine could be determined most accurately by incubating *Leuconostoc mesenteroides* P-60 for 5 days at 35° in a

TABLE III  
Results of Assays of Phenylalanine in Amino Acid Test Mixture 3\*

Amino acid mixture per tube	<i>Leuconostoc mesenteroides</i>			<i>Lactobacillus casei</i>		
	Titration volume of 0.111 N NaOH†	Phenylalanine found‡		Titration volume of 0.111 N NaOH†	Phenylalanine found‡	
		Per tube	Per ml. sample		Per tube	Per ml. sample
$\gamma$	ml.	$\gamma$	$\gamma$	ml.	$\gamma$	$\gamma$
250.7	7.19	2.44	4.88	9.07	2.30	4.60
501.5	9.80	4.72	4.72	11.25	4.79	4.79
752.2	12.10	7.25	4.83	13.23	7.55	5.03
1003.0	13.92	9.49	4.75	15.02	10.05	5.02
1253.7	15.85	12.25	4.90	16.39	12.40	4.96

\* The composition of the test mixture simulating silk fibroin was the same as that given in Paper XIX (21), except that the mixture contained 0.96 per cent of *l*(-)-phenylalanine (as *dl*-phenylalanine). The basal medium  $\times$  2 and 4 days incubation at 35° were employed with *Leuconostoc mesenteroides* and 3 days incubation at 35° was employed with *Lactobacillus casei*.

† The solutions in the six replicate tubes employed at each level of sample were mixed and the mixture titrated. The average value given per tube at each level of sample was calculated from these data.

‡ An average of 4.82  $\gamma$  of phenylalanine was found per ml. of amino acid test mixture. The average mean deviation from the mean at the different levels was 1.3 per cent. The recovery of phenylalanine was 99.5 per cent. The recoveries of phenylalanine in analogous experiments were 103 and 104 per cent (basal medium  $\times$  1.3, 4 days incubation), 105 and 111 per cent (basal medium  $\times$  1.3, 5 days incubation), 103 and 103 per cent (basal medium  $\times$  2, incubation 4 days), and 101 per cent (basal medium  $\times$  2, 5 days incubation).

§ An average of 4.88  $\gamma$  of phenylalanine was found per ml. of amino acid test mixture. The average mean deviation from the mean at the different levels was 3.1 per cent. The recovery of phenylalanine was 101.6 per cent in this experiment and 99 per cent in an analogous experiment.

basal medium with total amino acids at twice the level employed previously for the assay of histidine and lysine. Under these conditions the average mean deviations from the mean values (five levels of samples) averaged about 1 per cent, the assay values for phenylalanine in casein agreed closely, and the recoveries of phenylalanine in the amino acid test mixtures and the casein hydrolysates were approximately 100 per cent.

It appears significant that the accuracy with which phenylalanine could

be recovered from amino acid test mixtures decreased markedly as the percentage of phenylalanine decreased from 2.44 to 0.50 per cent and that the recoveries were unsatisfactory from such mixtures containing 0.17 per cent of this amino acid.<sup>3</sup> Since the accuracy of microbiological assays may vary inversely with the proportion of the amino acid in the sample,

TABLE IV  
*Results of Assay of Phenylalanine in Casein Hydrolysate*

Casein per tube (corrected for moisture and ash)	<i>Leuconostoc mesenteroides</i>			<i>Lactobacillus casei</i>		
	Titration vol- ume of 0.111 N NaOH*	Phenylalanine found†		Titration vol- ume of 0.0814 N NaOH*	Phenylalanine found†	
		Per tube	Casein (corrected for moisture and ash)		Per tube	Casein (corrected for moisture and ash)
$\gamma$	ml.	$\gamma$	per cent	ml.	$\gamma$	per cent
46.3	7.00	2.28	4.92	9.20	2.45	5.30
92.7	9.61	4.56	4.92	11.39	4.95	5.31
139.0	11.84	6.96	5.01	13.12	7.41	5.32
185.4	13.56	9.01	4.86	14.50	9.32	5.03
231.7	15.20	11.26	4.86	16.00	12.00	5.18
Average.....			4.91			5.23

\* The solutions in the six replicate tubes employed at each level of sample were mixed and the mixture titrated. The average value given per tube at each level of sample was calculated from these data.

† The basal medium  $\times 2$  and 5 days incubation were employed. The average mean deviation from the mean at the different levels was 0.9 per cent. The following corrected values for phenylalanine were found from comparable assays: 4.94 and 4.96 per cent (basal medium  $\times 1.3$ , 4 days incubation), 4.88 and 5.04 per cent (basal medium  $\times 1.3$ , 5 days incubation), 4.97 and 4.99 per cent (basal medium  $\times 2$ , 4 days incubation), and 4.91 per cent (basal medium  $\times 2$ , 5 days incubation).

‡ The average mean deviation from the mean at the different levels was 1.9 per cent. The basal medium  $\times 1$  and 3 days incubation were employed. The corrected value, 5.02 per cent, was found in a comparable assay.

there may be a limiting accuracy with which a given amino acid may be determined in a particular protein with a particular microorganism.

### *Phenylalanine in Casein*

The percentage of phenylalanine determined under the best conditions (basal medium<sup>4</sup>  $\times 2$ , 5 days incubation of *Leuconostoc mesenteroides* at

<sup>3</sup> Since the phenylalanine content of amino acid Test Mixture 2 (with phenylalanine omitted) was found by assay to be less than 0.01 per cent, contamination of these amino acids with phenylalanine could not be a significant factor.

<sup>4</sup> The expression "basal medium  $\times 2$ " signifies that the composition of the basal

35°) was 4.90 (4.89 and 4.91), corrected for the 6.21 per cent moisture and the 0.55 per cent ash reported previously (20). The average corrected value, 4.97 (4.89 to 5.05, range in six determinations) per cent, was obtained with *Leuconostoc mesenteroides* under the other stipulated conditions. The average corrected value found with *Lactobacillus casei* was 5.1 (5.1, 5.0, and 5.2) per cent.

It seems probable that the true value for phenylalanine in casein is

TABLE V  
Recovery of Phenylalanine Added to Casein Hydrolysate\*

Casein per tube (corrected for moisture and ash) (I)	l(-) Phenylalanine added per tube (II)	l(-) Phenylalanine					
		<i>Leuconostoc mesenteroides</i>			<i>Lactobacillus casei</i>		
		In casein per tube† (III)	Found per tube (IV)	Recovery‡ (V)	In casein per tube§ (VI)	Found per tube (VII)	Recovery   (VIII)
γ	γ	γ	γ	per cent	γ	γ	per cent
23.2	1.20	1.14	2.41	105.8	1.21	2.36	95.8
46.3	2.40	2.28	4.68	100.0	2.42	4.95	105.4
69.5	3.60	3.42	6.99	99.2	3.63	7.27	101.1
92.6	4.80	4.56	9.34	99.6	4.84	9.60	99.2
115.8	6.00	5.70	11.45	95.8	6.05	12.51	107.7
Average				100.1			101.8

\* The casein hydrolysate sample assayed was prepared to contain 2.40 γ of l(-)-phenylalanine per ml (added as twice the quantity of dl-phenylalanine) and 46.3 γ of hydrolyzed casein (corrected for moisture and ash) per ml. Volumes of this solution varying from 0.5 to 2.5 ml were taken for the assays. The solutions from the six replicate tubes at each level were mixed and titrated.

† Estimated on the basis of the 4.91 per cent of phenylalanine in casein given in Table IV. The basal medium × 2 and 5 days incubation were employed.

‡ Calculated from the relation,  $((IV - III)/II) \times 100 = \text{per cent recovery}$ . The recoveries of phenylalanine in comparable experiments were 103 per cent (basal medium × 1.3, 4 days incubation), 110 per cent (basal medium × 1.3, 5 days incubation), and 100 per cent (basal medium × 2, 4 days incubation).

§ Estimated on the basis of the 5.23 per cent of phenylalanine in casein given in Table IV. The unaltered basal medium and 3 days incubation were employed.

|| Calculated from the relation,  $((VII - VI)/II) \times 100 = \text{per cent recovery}$ .

5.0 ± 0.2 per cent. The uncorrected value, 4.77 per cent, has been reported recently by Albanese (22) who determined phenylalanine by the Kapeller-Adler (23) colorimetric procedure after eliminating three interfering amino

medium (Medium D, Table I, described in Paper XVIII (18)) has been modified to contain twice the stated concentrations of amino acids but the same concentrations of all other constituents. The expression "basal medium × 1.3" is employed in this paper in a corresponding sense.

acids. Tryptophane was destroyed by acid hydrolysis of the casein, histidine was removed by adsorption on permutit, and tyrosine was destroyed by oxidation with acid  $\text{KMnO}_4$  solution. It was shown that from 100 to 102 per cent of phenylalanine added to a mixture of histidine and tyrosine and to hydrolysates of casein and gelatin was recovered. It is

TABLE VI  
*Results of Assay of Phenylalanine in Silk Fibroin Hydrolysate*

Silk fibroin per tube (corrected for moisture and ash)	<i>Leuconostoc mesenteroides</i>			<i>Lactobacillus casei</i>		
	Titration volume of 0.111 N NaOH*	Phenylalanine found†		Titration volume of 0.111 N NaOH	Phenylalanine found†	
		Per tube	Silk fibroin (corrected for moisture and ash)		Per tube	Silk fibroin (corrected for moisture and ash)
$\gamma$	ml.	$\gamma$	per cent	ml.	$\gamma$	per cent
118.8	6.04	1.54	1.30	8.32	1.54	1.30
237.5	7.87	3.05	1.28	10.10	3.45	1.45
356.3	9.49	4.45	1.25	11.49	5.10	1.43
475.1	11.05	6.09	1.28	12.69	6.80	1.43
593.9	12.48	7.68	1.29	14.00	8.61	1.45
Average.....			1.28			1.41

\* The solutions from the six replicate tubes at each level of sample were mixed and titrated.

† The basal medium  $\times 2$  and 5 days incubation were employed. The average mean deviation from the mean at the different levels was 1.0 per cent. The following corrected values for phenylalanine were found from comparable assays: 1.29 per cent (basal medium  $\times 1.3$ , 4 days incubation), 1.37 per cent (basal medium  $\times 1.3$ , 5 days incubation), and 1.28 per cent (basal medium  $\times 2$ , 4 days incubation).

‡ The average mean deviation from the mean at the different levels was 3.3 per cent. The basal medium  $\times 1$  and 3 days incubation were employed.

of interest that Kapeller-Adler (23) found values from 4.5 to 5.3 per cent (presumably uncorrected) for phenylalanine in casein essentially by the same procedure except that histidine was removed as its phosphotungstate. Kuhn and Desnuelle (24) determined phenylalanine by the Kapeller-Adler method in an acid hydrolysate of Hammarsten casein (15.66 per cent nitrogen after drying 24 hours at  $80^\circ$ ) from which the basic and dicarboxylic amino acids had been removed by electrodialysis. Although only 4.1 per cent of phenylalanine was found, about 17 per cent of the nitrogen in the casein sample was lost through formation of humin, conversion to ammonia, and adsorption on the  $\text{BaSO}_4$  precipitates.

It is difficult to understand why the values (Table VIII) for phenylalanine

determined by colorimetric analysis of casein hydrolyzed with NaOH, HCl, H<sub>2</sub>SO<sub>4</sub>, HI, and mixtures of some of these acids should be so divergent and why the values for phenylalanine in casein hydrolyzed for different times with a given acid or base should fluctuate so widely. Although Schein and Berg (38) found that "neither destruction nor racemization

TABLE VII  
Recovery of Phenylalanine Added to Silk Fibroin Hydrolysate\*

Silk fibroin per tube (corrected for moisture and ash) (I)	l(-)-Phenylalanine added per tube (II)	l(-)-Phenylalanine					
		<i>Leuconostoc mesenteroides</i>			<i>Lactobacillus casei</i>		
		In silk fibroin per tube† (III)	Found per tube (IV)	Recovery‡ (V)	In silk fibroin per tube† (VI)	Found per tube (VII)	Recovery§ (VIII)
γ	γ	γ	γ	per cent	γ	γ	per cent
59.4	1.20	0.76	1.91	95.8	0.84	2.13	107.5
118.8	2.40	1.52	3.76	93.3	1.67	4.30	109.6
178.2	3.60	2.28	5.64	93.3	2.51	6.80	119.2
237.5	4.80	3.04	7.63	95.6	3.35	8.57	108.8
297.0	6.00	3.80	9.46	94.3	4.19	10.80	110.2
Average .....				94.5			111.1

\* The silk fibroin hydrolysate sample assayed was prepared to contain 2.40 γ of l(-)-phenylalanine per ml. (added as twice the quantity of dl-phenylalanine) and 118.8 γ of hydrolyzed silk fibroin (corrected for moisture and ash) per ml. Volumes of this solution varying from 0.5 to 2.5 ml. were taken for the assays. The solutions from the six replicate tubes at each level were mixed and titrated.

† Estimated on the basis of the 1.28 per cent of phenylalanine in silk fibroin given in Table VI. The basal medium × 2 and 5 days incubation were employed.

‡ Calculated from the relation,  $((IV - III)/II) \times 100 = \text{per cent recovery}$ . The recoveries of phenylalanine in comparable experiments were 97.1 per cent (basal medium × 1.3, incubation time 4 days), 96.2 per cent (basal medium × 1.3, incubation time 5 days), and 99.6 per cent (basal medium × 2, incubation time 4 days).

§ Estimated on the basis of the 1.41 per cent of phenylalanine in silk fibroin given in Table VI. The unaltered basal medium and 3 days incubation were employed.

|| Calculated from the relation,  $((VII - VI)/II) \times 100 = \text{per cent recovery}$ .

of the basic amino acids occurs until the minimum time required [18 hours at 120°] for the acid [8 N H<sub>2</sub>SO<sub>4</sub>] hydrolysis of casein has been greatly exceeded," it may be that phenylalanine behaves in an entirely different manner under these conditions. It is recognized that amino acids differ markedly in stability towards acids and bases and that additional studies should be made of phenylalanine and other amino acids under different experimental conditions. This problem is under investigation in the authors' laboratory.



TABLE VIII  
Percentage of Phenylalanine in Casein (Literature Values)\*

Source of casein sample†	Hydrolysis procedure	Analytical method	Phenylalanine <i>per cent</i>	Reference No.
Harris.....	5 N NaOH for 5 hrs. at 125°	K-A‡	6.3§	31
Labco.....	Same	" ‡	4.8§	31
" hydrolyzed ..	"	" ‡	5.6,§ 6.2§	31
Difco " ..	"	" ‡	4.3,§ 4.6§	31
Source not given..	"	" ‡	5.8	32
" " " ...	8 N H <sub>2</sub> SO <sub>4</sub> for 2 hrs. at 125°	" ‡	3.9	33
" " " ...	20% HCl-HCOOH for 2 hrs. at 125°	" ‡	4.9	33
" " " ...	Concentrated HI for 2 hrs. at 125°	" ‡	4.3	33
" " " ...	20% HCl for 2 hrs. at 125°	" ‡	5.0	33
" " " ...	5 N NaOH for 2 hrs. at 125°	" ‡	5.8	33
" " " ...	20% HCl for 2, 4, 8, 18 hrs.	"	5.03, 4.39, 4.81, 3.93	34
" " " ...	5 N NaOH for 2, 4, 6, 18 hrs.	"	4.97, 4.73, 5.71, 5.52	34
" " " ...	7 " H <sub>2</sub> SO <sub>4</sub> " 2, 4, 8, 18 "	"	3.89, 5.69, 5.46, 4.71	34
Labco, vitamin-free.....	5 " NaOH " 24 hrs.	" ‡	3.99¶	35
Authors' preparation**.....	25% H <sub>2</sub> SO <sub>4</sub> " 20 "	" ‡	5.5††	36
Hammarsten, fat-free.....	H <sub>2</sub> SO <sub>4</sub>	Benzoic acid‡‡	2.68, 3.04, 3.2, 3.4	37
H. B. Vickery.....	6 N HCl for 24 hrs.	Microbiological§§	3.70	15

\* In 1919, Foreman (25) isolated 3.9 per cent of phenylalanine as the hydrochloride from an ester fraction of a sulfuric acid hydrolysate of casein. Lower percentages of phenylalanine were isolated earlier by Fischer (26), Fischer and Abderhalden (27), and Osborne and Guest (28) essentially by the same method. Boyd (29) isolated 2.2 per cent of phenylalanine as its hydantoin and Baptist and Robson (30) isolated 0.3 per cent of phenylalanine as its picrolonate.

† No physical or chemical characteristics of the casein samples were reported by the authors quoted other than those described in the foot-notes to this table.

‡ The Kappeler-Adler (23) colorimetric procedure.

§ Calculated for casein containing 16 per cent nitrogen.

|| Modified Kappeler-Adler colorimetric procedure.

¶ Average of several determinations (no values listed) corrected for moisture and ash. Tryptophane was removed as its HgSO<sub>4</sub> complex and phenylalanine determined in the mercury-free filtrate. Recovery of phenylalanine added to a mixture containing ten amino acids was 102.5 per cent.

TABLE VIII—*Concluded*

\*\* Precipitated from milk at the isoelectric point of casein.

†† Corrected for moisture and ash.

‡‡ Gravimetric determination of benzoic acid formed by oxidation of casein with potassium dichromate.

§§ *Lactobacillus arabinosus* 17-5.

||| Recovery of phenylalanine added to casein 92.5 (74 to 110) per cent.

### *Phenylalanine in Silk Fibroin*

On the whole the assays of phenylalanine in silk fibroin hydrolysates and the recoveries of this amino acid from these preparations were somewhat less accurate than those obtained with casein. These results were not unexpected in view of the relatively low percentage of phenylalanine and the unusual distribution of amino acids in this protein.

The phenylalanine content of silk fibroin determined under the most satisfactory experimental conditions (assay with *Leuconostoc mesenteroides* incubated 5 days at 35° with the amino acids  $\times 2$  in the basal medium) was found to be 1.28 per cent, corrected for the 5.68 per cent moisture and the 0.25 per cent ash reported previously (20). The average corrected value, 1.31 (1.29, 1.37, and 1.28) per cent, was obtained with *Leuconostoc mesenteroides* under the other stipulated conditions. The corrected value, 1.41 per cent, was obtained with *Lactobacillus casei*. Abderhalden and coworkers (39-47) found that silk fibroin prepared from silk of different sources contained from 0.6 to 1.6 per cent of phenylalanine and other investigators (48-51) have reported values ranging from 0.25 to 1.3 per cent.

### SUMMARY

Microbiological procedures have been described for the determination of phenylalanine in protein hydrolysates with two microorganisms, *Leuconostoc mesenteroides* P-60 and *Lactobacillus casei*. Closely agreeing values for the percentage of phenylalanine in casein and in silk fibroin were found by assays with these organisms. The corrected percentages of phenylalanine in casein and silk fibroin, respectively, were found to be 4.91 and 1.28 per cent under the conditions considered to be most reliable (assay with *Leuconostoc mesenteroides* incubated for 5 days at 35° in a basal medium identical with that reported previously (18) for the determination of lysine and histidine except that the concentration of total amino acids was doubled).

It is considered that the probable true value for phenylalanine in casein is  $5.0 \pm 0.2$  per cent, since data of high accuracy were obtained in the recovery of phenylalanine from three amino acid test mixtures of different composition and from casein hydrolysates. This figure is in approximate

agreement with the values obtained by investigators who employed what appeared to be dependable modifications of the Kapeller-Adler colorimetric procedure. It is recognized, however, that the extent to which phenylalanine is destroyed or altered during hydrolysis of casein must be measured before the true value for phenylalanine in this protein can be determined with certainty.

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# INVESTIGATIONS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

## XXIV. THE AMINO ACID REQUIREMENTS OF *LACTOBACILLUS FERMENTI* 36\*

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Reasonably satisfactory procedures for the determination of the majority of the amino acids with *Lactobacillus arabinosus*, *Lactobacillus casei*, *Lactobacillus delbrückii*, and *Leuconostoc mesenteroides* and the assay with these microorganisms of purified proteins (1-9), foodstuffs (6-8, 10-14), blood fractions (15, 16), animal muscle and organ proteins (12, 17, 18), alcoholic fermentation products (19), and bacterial cells (20) have been reported. Microbiological methods are not available for the assay of some amino acids and there is need for improved procedures for the assay of others. For this reason, the amino acid and other nutritional requirements of *Lactobacillus fermenti* 36 were investigated and the potentialities of this microorganism for amino acid assays were determined.

*Lactobacillus fermenti* 36, a strain of mannitol-forming bacteria, was first isolated by Stiles, Peterson, and Fred (21) in 1925 from fermenting cereal infusions. The growth requirements of this microorganism have been studied recently by Sarett and Cheldelin (22, 23) and a microbiological procedure for thiamine has been described by these investigators.

The nutritional requirements of *Lactobacillus fermenti* 36 were studied according to the plan adopted in comparable investigations of *Leuconostoc mesenteroides* P-60 (9, 24). Acid production was measured at a series of arbitrarily chosen levels of amino acids and other nutrients, a standard curve for each essential amino acid was constructed from the titration data, and the minimal concentration of each essential amino acid required to give the maximal titration was estimated from its standard curve. In addition, experiments with multiple media were performed to determine the levels of glucose, salts, purines and pyrimidines, vitamins, and amino acids at which the standard curves for a particular amino acid were nearly coincident and were approximately linear over the segment to be employed for assay

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TABLE I  
Composition of Basal Media\*

Constituent	Medium A	Medium B	Medium C
	<i>mg. per l.</i>	<i>mg. per l.</i>	<i>mg. per l.</i>
<i>dl</i> -Alanine.....	200	400	400
<i>l</i> (+)-Arginine monohydrochloride.....	50	160	160
Asparagine (natural).....	400	200	200
<i>l</i> (-)-Cystine.....	100	200	200
<i>l</i> (+)-Glutamic acid.....	200	240	240
Glycine.....	100	200	200
<i>l</i> (-)-Histidine monohydrochloride mono- hydrate.....	50	90	90
<i>l</i> (-)-Hydroxyproline.....	100	200	200
<i>dl</i> -Isoleucine.....	200	400	400
<i>l</i> (-)-Leucine.....	200	200	200
<i>l</i> (+)-Lysine monohydrochloride.....	100	200	200
<i>dl</i> -Methionine.....	100	200	200
<i>dl</i> -Norleucine.....	100	200	200
<i>dl</i> -Norvaline.....	100	200	200
<i>dl</i> -Phenylalanine.....	100	200	200
<i>l</i> (-)-Proline.....	100	200	200
<i>dl</i> -Serine.....	50	200	200
<i>dl</i> -Threonine.....	200	200	200
<i>l</i> (-)-Tryptophane.....	50	32	32
<i>l</i> (-)-Tyrosine.....	25	100	100
<i>dl</i> -Valine.....	200	400	400
Glucose.....	20 (gm.)	20 (gm.)	30 (gm.)
Adenine sulfate.....	12	12	24
Guanine hydrochloride.....	12	12	24
Uracil.....	12	12	24
Xanthine.....	0	0	24
Sodium acetate.....	12 (gm.)	12 (gm.)	10 (gm.)
NH <sub>4</sub> Cl.....	6 "	6 "	3 "
KH <sub>2</sub> PO <sub>4</sub> .....	500	500	500
K <sub>2</sub> HPO <sub>4</sub> .....	500	500	500
MgSO <sub>4</sub> ·7H <sub>2</sub> O.....	200	200	200
NaCl.....	5 (gm.)	5 (gm.)	0
FeSO <sub>4</sub> ·6H <sub>2</sub> O.....	10	10	10
MnSO <sub>4</sub> ·4H <sub>2</sub> O.....	10	10	10
Thiamine hydrochloride.....	1	1	1
Pyridoxine.....	1.6	1.6	1.6
Calcium <i>dl</i> -pantothenate.....	2	2	2
Riboflavin.....	2	2	2
Nicotinic acid.....	2	2	2
Biotin.....	5 (γ)	5 (γ)	5 (γ)
Folic acid.....	2.5 "	2 "	2 "
<i>p</i> -Aminobenzoic acid.....	0.1	0.1	0.1

TABLE I—*Concluded*

\* The sources and specifications of the listed nutrients were the same as those given previously (24). The composition of the medium was essentially the same as that of Sarett and Cheldelin (23) except that amino acids instead of acid-hydrolyzed casein, about one-half the concentration of sodium acetate, 1.5 times the concentration of glucose, and from 1 to 20 times the concentration of vitamins were employed. Xanthine was added to the present authors' basal medium. The basal media as prepared contained 6 times the concentration of the constituents given in Table I. The concentration of constituents of the basal media present in the final assay solutions was the same as that shown in Table I. Equivalent quantities of cystine and cysteine hydrochloride were used interchangeably. The temperature was 35° and the incubation time 3 days in all experiments except those in which other conditions are specified.

purposes. A basal medium with nutrients at the minimal levels found to give the desired response of the microorganism was considered to be satisfactory for assay purposes.

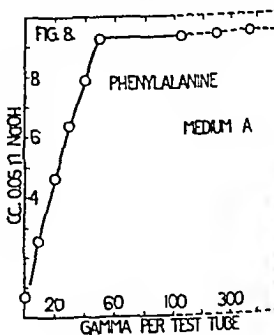
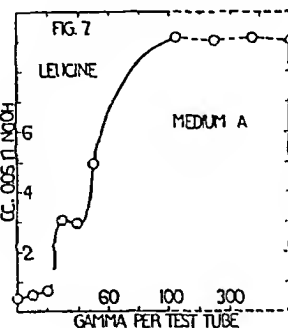
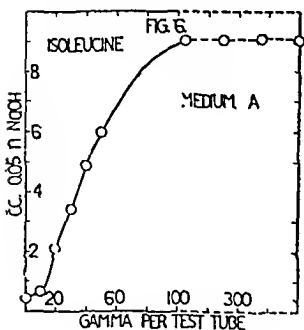
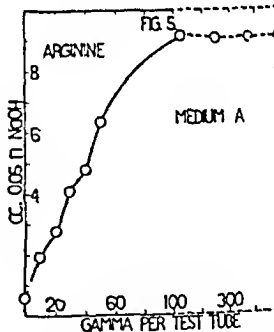
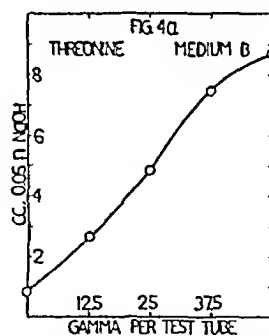
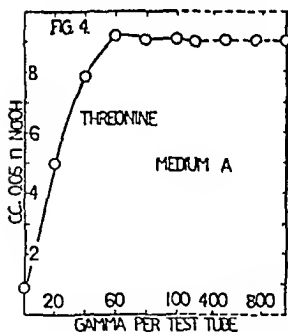
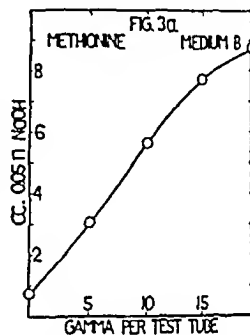
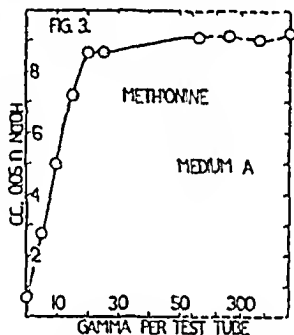
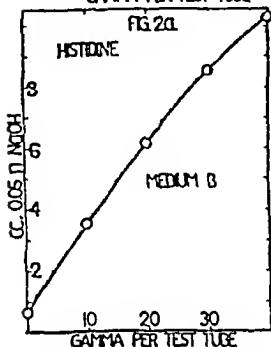
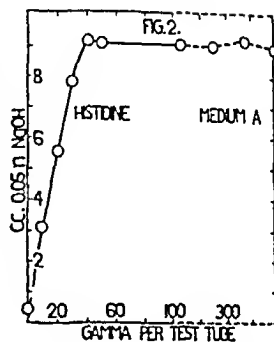
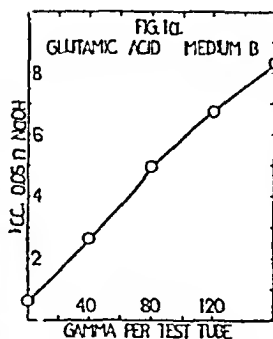
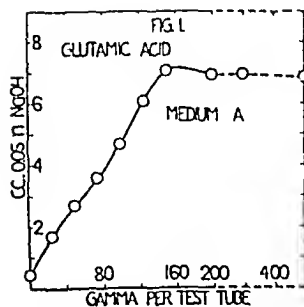
According to the authors' experience, acid production is approximately maximal in a medium which is satisfactory for an amino acid assay. On the other hand it has been found that, contrary to the view of some workers (25), maximal acid production does not necessarily insure the adequacy of a medium. Neither acid production nor the slope of the standard curve, *per se*, is considered to be the most significant determinant of a medium's assay potentialities. One of the most critical factors is believed to be the capacity of the medium to resist, over as wide a range of concentrations as possible, the stimulatory or inhibitory influence of amino acids and other substances which may be introduced during an assay.

#### EXPERIMENTAL

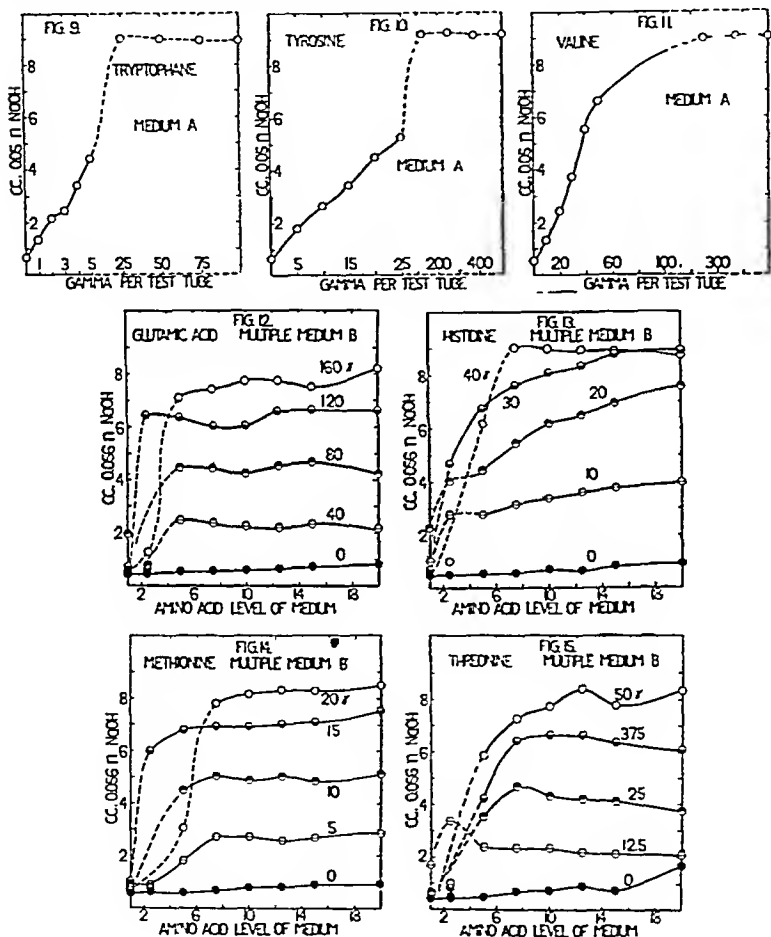
The microorganism, *Lactobacillus fermenti* 36, American Type Culture Collection, No. 9338, was obtained from the University of Oregon through the courtesy of Dr. V. H. Cheldelin. The culture was carried on Bactotomato juice agar (Difco), since it grew poorly on yeast-dextrose agar (Difco). The assay technique was that described previously (24). Solutions of the standard amino acid, the samples, the basal media, and suspensions of the inocula were transferred to 6 inch test-tubes with the aid of a Brewer automatic pipette (Baltimore Biological Laboratories). The composition of the basal media is given in Table I.

The types and proportions of the substances in the basal medium (Medium A, Table I) selected arbitrarily for the initial study of the nutritional requirements of *Lactobacillus fermenti* 36 were nearly the same as those employed previously (2). Simultaneous growth experiments were carried out at five levels of histidine (Medium B, Table I) and two incubation





temperatures. It was concluded that 35° is a satisfactory incubation temperature, since acid production increased only slowly after 72 hours and growth was more rapid than at 28°.



It is considered important that the centrifuged inoculum be suspended in a volume of saline approximately equal to that of the cell suspension. Although satisfactory standard curves (histidine) were obtained with undiluted inocula grown in 5 ml. final volumes per 6 inch test-tube either in

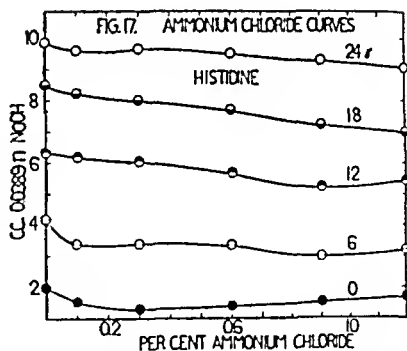
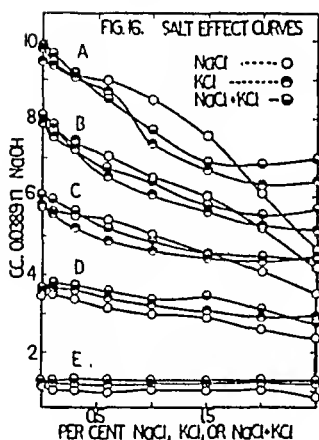


FIG. 16. The micrograms of histidine employed were as follows: Curves A, 40; Curves B, 30; Curves C, 20; Curves D, 10; and Curves E, 0.

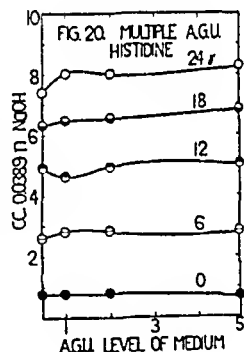
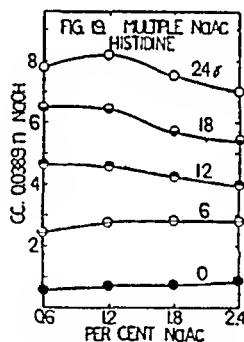
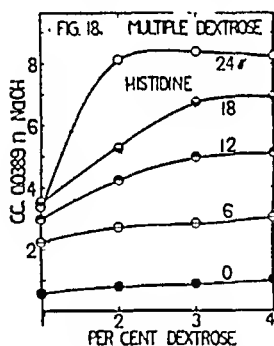
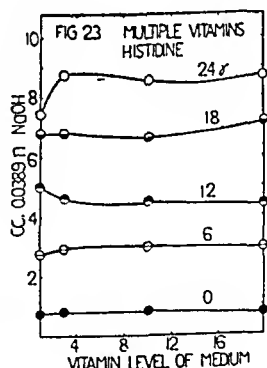
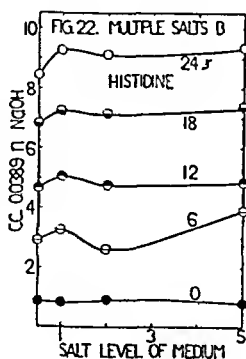
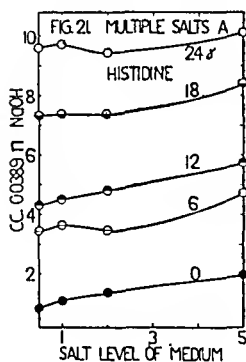


FIG. 20. A. = adenine; G. = guanine; U. = uracil.



Medium A or Medium C, inocula diluted 1:100 did not grow in Medium A with up to 6 days incubation. Inocula diluted 1:100 did not grow in Medium C after 2 days incubation and growth was slow and erratic up to 6

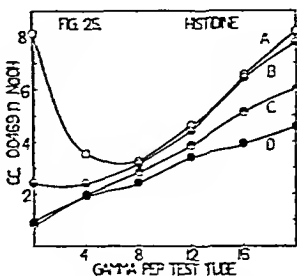
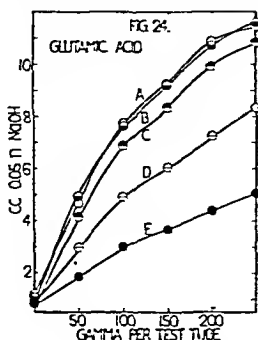


FIG. 24. Days of incubation time were as follows: Curve A, 6; Curve B, 5; Curve C, 3; Curve D, 2; and Curve E, 1.

FIG. 25. Days of incubation time were as follows: Curve A, 4; Curve B, 3; Curve C, 2; and Curve D, 1.

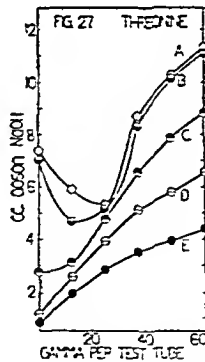
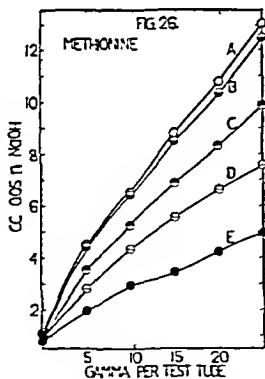


FIG. 26. Days of incubation time were as follows: Curve A, 6; Curve B, 5; Curve C, 3; Curve D, 2; and Curve E, 1.

FIG. 27. Days of incubation time were as follows: Curve A, 6; Curve B, 5; Curve C, 3; Curve D, 2; and Curve E, 1.

days incubation. These observations coincide with those of Sarett and Cheldelin (23) who obtained poor growth of dilute inocula unless alkali-treated peptone was added to the medium. These results suggest that alkali-treated peptone contains a growth factor (or factors) not present in

purified casein or the present authors' synthetic medium. Curves showing the experimental results are given in Figs. 1 to 27.

#### DISCUSSION

It was found with Medium A, Table I, that the amino acids arginine, glutamic acid, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophane, tyrosine, and valine were essential nutrients and that the other amino acids listed were auxiliary or non-essential substances. Standard curves showing the response of the microorganisms to different levels of the eleven essential amino acids are given in Figs. 1 to 11.

Medium B, Table I, was prepared by adjusting the concentration of the essential amino acids to the levels found to give maximal acid production in the preliminary experiments with Medium A. The changes in concentration of the other amino acids stipulated in Medium B, Table I, were made arbitrarily although they were suggested by the unpublished experimental data obtained in the preliminary experiments with Medium A. Standard curves were obtained showing the response of the microorganism with Medium B to different levels of the four essential amino acids, glutamic acid, histidine, methionine, and threonine.<sup>1</sup> It may be noted that the linear portion of these curves (Figs. 1, *a* to 4, *a*) has been extended somewhat beyond the limits observed in the comparable curves with Medium A. It was assumed, therefore, that Medium B would be more satisfactory for assay purposes than Medium A.

Experiments were next performed to determine the minimal level of total amino acids in Medium B required to give maximal acid production at each of several concentrations of each of the four amino acids, glutamic acid, histidine, methionine, and threonine. The response of the microorganism under these conditions is shown in Figs. 12 to 15. Eight media with total amino acids ranging from 0.125 to 2.5 times (given as Levels 1 and 20 respectively in Figs. 12 to 15) the concentrations shown in Medium B, Table I, were employed. Standard curves were obtained at five concentrations of each of the four essential amino acids. These concentrations were 0 to 160  $\gamma$  for glutamic acid, 0 to 40  $\gamma$  for histidine, 0 to 20  $\gamma$  for methionine, and 0 to 50  $\gamma$  for threonine. It was concluded that 1  $\times$  the concentration (equivalent to Level 8 of Figs. 12 to 15) of total amino acids was the minimal level which would be satisfactory for the assay of these four amino acids under the described experimental conditions.

<sup>1</sup> Experiments with multiple media in which the concentrations of total amino acids varied from 1 to 2.5 times those given in Medium C, Table I, were carried out with phenylalanine. It was observed that the standard curves were depressed markedly with increasing concentration of total amino acids. It appeared, therefore, that phenylalanine could not be determined satisfactorily with *Lactobacillus fermenti* 36 under the described experimental conditions.

The nutritional adequacy of Medium B was further investigated with total amino acids (except histidine) present in the concentrations shown in Table I, but with multiple concentrations of other nutrients. Curves showing the response of the microorganism at five concentrations of histidine up to 24 or 40  $\gamma$  are shown in Figs. 16 to 23. The following observations were made: (a) Acid production was strongly inhibited (Fig. 16) up to 2.5 per cent concentrations of NaCl, KCl, and an equal mixture of these salts, although NaCl was most strongly and the mixture least inhibitory. It may be inferred, therefore, that sodium and potassium salts are interrelated in the metabolism of *Lactobacillus fermenti* 36 and that all solutions should be adjusted to the same concentration of NaCl and of KCl to compensate for any stimulatory or inhibitory effects. (b) High blanks and erratic response of the microorganism resulted (Fig. 17) when  $\text{NH}_4\text{Cl}$  was omitted from the basal medium and acid production was inhibited regularly up to 1.2 per cent concentration of this salt. (c) The increase in acid production (Fig. 18) was marked when glucose was increased from 1 to 2 per cent but the increase in acid was only slight when glucose was increased from 2 to 3 per cent. Acid production was not increased further when glucose was increased from 3 to 4 per cent. (d) There was no significant change in acid production (Fig. 19) when the concentration of sodium acetate was increased from 0.6 to 2.4 per cent. (e) Acid production was increased only slightly when the concentration of adenine, guanine, and uracil was doubled (Fig. 20) but no additional effect was noted up to the 5-fold level. There was a small increase in acid production (curves not given) at the 2-fold level of adenine, guanine, and uracil when xanthine at the same level was added to the medium. (f) There was a gradual but parallel increase in acid production (Fig. 21) up to a 5-fold level of the phosphate buffer salts\* (Salts A). (g) Acid production was not affected (Fig. 22) by increasing 5 times the concentration of Salts B (magnesium, manganese, and iron sulfates) but it was slightly decreased at 0.5 concentration of these non-buffer salts. (h) Acid production was unchanged (Fig. 23) up to 2 (equivalent to Level 20) times the concentration of total vitamins but it was decreased slightly at 0.1 (equivalent to Level 1) concentration of vitamins.

Good growth of *Lactobacillus fermenti* 36 and maximal acid production were observed in media containing no histidine, relatively low concentrations of sodium acetate and NaCl, and from 0 to 0.3 per cent of  $\text{NH}_4\text{Cl}$ .

\* This effect was observed by Sarett and Cheldelin (23). It is believed to be explained by the maintenance of a nearly constant but relatively high pH in solutions containing relatively high concentrations of buffer salts. Under these conditions, acid production would be less inhibited than in solutions containing relatively low concentrations of buffer salts.

When 10  $\gamma$  of histidine per tube were added, acid production decreased to about 40 per cent of the maximal but above 10  $\gamma$  of histidine it returned approximately to maximal. At 0.6 per cent  $\text{NH}_4\text{Cl}$  and 1.2 per cent sodium acetate the blank titrations were low even in the absence of  $\text{NaCl}$ . Apparently, *Lactobacillus fermenti* 36 is able to synthesize histidine at relatively low concentrations of salts, while at higher concentrations of salts or relatively low concentrations of histidine this synthesis is partly inhibited.

That this effect bears a relation to the time of incubation was shown in other experiments with glutamic acid, histidine, methionine, and threonine. Although no synthesis of glutamic acid (Fig. 24) or of methionine (Fig. 26) occurred up to 5 days, synthesis of histidine (Fig. 25) and of threonine (Fig. 27) was appreciable in 3 days and was marked in 4 or 5 days. It is apparent, therefore, that incubation times longer than 2 days should not be employed for the microbiological determination of histidine or threonine under the described experimental conditions. It is considered probable that there may be particular experimental conditions under which a particular amino acid may be synthesized by any microorganism. In view of this possibility, it would seem to be fortuitous that it has been possible, in a relatively short time, to devise reasonably satisfactory procedures for the determination of so many amino acids by microbiological methods.

It has been reported (21) that sucrose is better utilized by *Lactobacillus fermenti* than glucose. It was found in the present experiments that this microorganism did not grow on the described basal medium containing sucrose in place of glucose. This failure may possibly be attributed to the absence of small amounts of intermediate products (26, 27), essential for the initiation of fermentation, which are formed when media containing glucose are autoclaved.

Medium C, Table I, was prepared after consideration of the data recorded in the curves shown in Figs. 12 to 23. It has been assumed that this medium is satisfactory for the microbiological determination of the amino acids which are essential for the growth of *Lactobacillus fermenti* 36 and experiments, designed to test the validity of this hypothesis for histidine, are described in Paper XXV.

#### SUMMARY

Amino acids essential for the growth of *Lactobacillus fermenti* 36 have been determined. The response of this microorganism to glutamic acid, histidine, methionine, and threonine at different levels of total amino acids and its response to histidine at different levels of  $\text{NaCl}$ ,  $\text{KCl}$ , equal mixtures of  $\text{NaCl}$  and  $\text{KCl}$ ,  $\text{NH}_4\text{Cl}$ , glucose, sodium acetate, purines and pyrimidines, phosphate buffer salts, non-buffer salts, and vitamins has been measured in terms of acid production. It has been shown that histidine and threonine, but not glutamic acid and methionine, are syn-

thesized by *Lactobacillus fermenti* 36 after 2 days incubation under the described experimental conditions. It has been concluded that the basal medium and the experimental conditions established on the basis of the described experiments may be satisfactory for the determination of some amino acids with *Lactobacillus fermenti* 36.

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# INVESTIGATIONS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

## XXV. THE DETERMINATION OF HISTIDINE IN PROTEIN HYDROLYSATES WITH *LACTOBACILLUS FERMENTI* 36\*

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(Received for publication, September 4, 1945)

The purpose of the present experiments was to test the adequacy of the basal medium described in the foregoing paper (1) for the determination of histidine in protein hydrolysates with *Lactobacillus fermenti* 36. It was desired, also, to obtain additional evidence concerning the probable true percentage of histidine in casein. The corrected value (3.1 per cent) found previously (2) was in good agreement with that (3.1 per cent) reported by Albanese (3) but it was 6 per cent higher than the percentage (2.9) found by Saidel and Brand (4) and 24 per cent higher than the percentage (2.5) found by Vickery and Winternitz (5).

### EXPERIMENTAL

The assay technique (6) and a modification of the basal medium (Medium C, Table I) described previously (1) were employed. Final volumes of 5 ml. per 6 inch test-tube were utilized in studying the nutritional requirements of *Lactobacillus fermenti* 36 (1) but it was found in the present work that growth was superior with final volumes of 3 ml. per 4 inch test-tube. The latter conditions are also more convenient and more economical of chemicals than the former. The standard (histidine) was run at fifteen levels, the amino acid test mixtures were run at five levels, and six tubes were used at each level of standard and sample. After the tubes had been incubated, it was found necessary to steam them for 10 minutes to remove excess gas (presumably largely carbon dioxide) which caused fading of the color at the end-point in the titrations.

Relatively concentrated inocula were employed in the experiments described in the foregoing paper (1), since it was observed that, at dilutions of 1:100, growth of *Lactobacillus fermenti* 36 in Medium A or Medium C was poor or did not occur even after incubation for 6 days. It was considered

\* For Paper XXIV in this series see Dunn *et al.* (1). This work was aided by grants from the Gelatin Products Company, Merck and Company, Inc., the Nutrition Foundation, Inc., Schering and Glatz, and the University of California. The authors are indebted to H. Block, W. Frankl, B. Merrifield, and L. B. Rockland for valuable suggestions and technical assistance.

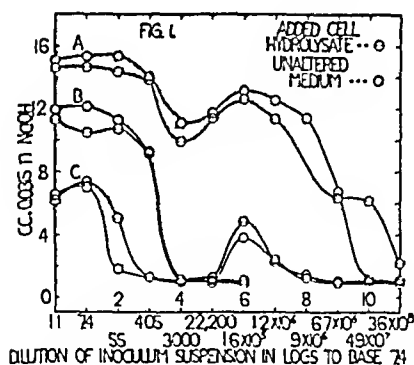


FIG. 1. The incubation time was as follows: Curves A, 68 hours; Curves B, 44 hours; and Curves C, 20 hours. The figures above the base-line refer to logarithms to the base 7.4 and those below the base-line refer to dilutions of the inoculum suspension corresponding to the indicated logarithmic values.

TABLE I  
Results of Assays of Histidine in Amino Acid Test Mixture 1\*

Amino acid mixture per tube	1 day incubation time				2 days incubation time			
	Titration volume of 0.0318 N NaOH per tube		Histidine found†		Titration volume of 0.0318 N NaOH per tube		Histidine found†	
	Range‡	Average	Per tube	Per ml. sample	Range‡	Average	Per tube	Per ml. sample
$\gamma$	ml.	ml.	$\gamma$	$\gamma$	ml.	ml.	$\gamma$	$\gamma$
281.4	2.16-2.30	2.24	5.03	25.15	2.94-3.03	2.98	4.90	24.50
562.6	2.95-3.18	3.05	10.52	26.30	4.59-4.78	4.68	10.40	26.00
843.9	3.62-3.80	3.71	14.75	24.58	6.20-6.29	6.24	15.40	25.67
1125.2	4.19-4.40	4.28	19.55	24.44	7.31-7.50	7.40	20.42	25.52
1406.5	4.70-4.89	4.80	24.10	24.10	8.39-8.81	8.56	25.75	25.75
Average.....				24.91				25.49

The solution analyzed contained 25.00  $\gamma$  of histidine per ml. The recovery of histidine was 99.8 per cent (1 day's incubation) and 102.2 per cent (2 days incubation).

\* The composition of the test mixture simulating casein was the same as that given in a previous paper (7) except that the mixture contained 1.77 per cent of histidine (as *l*(-)-histidine monohydrochloride monohydrate). The basal medium was Medium C, Table I (1), with total amino acids 1.5 times the stated concentrations.

† Six replicate tubes at each level of sample.

‡ The average mean deviations from the mean at the different levels were 2.5 per cent (1 day's incubation) and 1.5 per cent (2 days incubation).

desirable, therefore, to determine the dilution of inocula which might be most satisfactory for assay purposes. An inoculum, incubated for 21 hours, was centrifuged and the cells were suspended in an equal volume of sterile

saline. The transmission of this suspension was found to be 74 per cent and its optical density was 0.133, compared with distilled water, by measurement with a photoelectric colorimeter (Lumetron) fitted with a light diffusion screen. 1 drop (about 0.05 ml.) of dilutions of this suspension up to 1:3,640,000,000 was added to separate 3.0 ml. volumes of Medium C, Table I (1), in 4 inch test-tubes and the tubes were incubated at 35-37° for periods ranging from 20 to 68 hours. In some experiments, approxi-

TABLE II  
*Results of Assays of Histidine in Amino Acid Test Mixture 2\**

Amino acid mixture per tube	1 day incubation time				2 days incubation time			
	Titration volume of 0.0318 N NaOH per tube		Histidine found†		Titration volume of 0.0318 N NaOH per tube		Histidine found†	
	Range‡	Average	Per tube	Per ml. sample	Range‡	Average	Per tube	Per ml. sample
$\gamma$	ml.	ml.	$\gamma$	$\gamma$	ml.	ml.	$\gamma$	$\gamma$
404	2.09-2.11	2.10	4.15	20.75	2.61-2.81	2.70	4.00	20.00
808	2.71-2.88	2.78	8.75	21.87	3.76-3.88	3.82	7.90	19.75
1212	3.20-3.60	3.41	12.55	20.92	5.26-5.40	5.35	12.25	20.41
1616	3.69-4.20	3.86	15.85	19.81	6.49-6.62	6.56	16.80	21.00
2020	4.20-4.59	4.38	20.40	20.40	7.26-7.60	7.47	20.80	20.80
Average.	.....	.....		20.75				20.39

The solution analyzed contained 20.0  $\gamma$  of histidine per ml. The recovery of histidine was 103.8 per cent (1 day's incubation) and 102.0 per cent (2 days incubation).

\* Test Mixture 2 contained 100 mg. per ml. of each of the amino acids given in Test Mixture 1 except that it contained 1.00 per cent of histidine (as l(-)-histidine monohydrochloride monohydrate). The basal medium was Medium C, Table I (1), with total amino acids 1.5 times the stated concentrations.

† Six replicate tubes at each level of sample.

‡ The average mean deviations from the mean at the different levels were 2.6 per cent (1 day's incubation) and 2.0 per cent (2 days incubation).

mately 100  $\gamma$  of *Lactobacillus fermenti* 36 cells, hydrolyzed by refluxing them for 20 hours with 8 N HCl, were added per ml. of basal medium. Acid production was measured by titration with 0.0315 N NaOH. The titration curves are shown in Fig. 1.

It is evident that acid production decreased sharply and irregularly with dilutions of inocula beyond 1:7 or 1:50 and that it decreased to a low constant level at 1:400, 1:3000, and 1:500,000,000 dilutions of inocula in media incubated for 20, 44, and 68 hours, respectively. That growth and acid production occurred at higher dilutions of inocula in 4 inch than

TABLE III  
Results of Assays of Histidine in Amino Acid Test Mixture 3\*

Amino acid mixture per tube	1 day incubation time				2 days incubation time			
	Titration volume of 0.0310 N NaOH per tube		Histidine found†		Titration volume of 0.0310 N NaOH per tube		Histidine found†	
	Range†	Average	Per tube	Per ml. sample	Range†	Average	Per tube	Per ml. sample
$\gamma$	ml.	ml.	$\gamma$	$\gamma$	ml.	ml.	$\gamma$	$\gamma$
1253.2	1.98-2.01	1.98	3.73	18.65	2.51-2.70	2.64	3.72	18.50
2506.4	2.56-2.65	2.59	6.98	17.45	3.54-3.94	3.76	6.92	17.30
3759.6	3.16-3.30	3.22	10.28	17.13	4.78-5.41	4.96	10.42	17.37
5012.8	3.60-3.89	3.79	13.59	16.99	5.67-5.98	5.80	13.00	16.25
6266.0	4.13-4.40	4.30	17.43	17.43	7.14-7.88	7.52	18.90	18.90
Average.....				17.53				17.66

The solution analyzed contained 17.8  $\gamma$  of histidine per ml. The recovery of histidine was 98.5 per cent (1 day's incubation) and 99.0 per cent (2 days incubation).

\* The composition of the test mixture simulating silk fibroin was the same as that given in a previous paper (8) except that it contained 0.284 per cent of histidine (as L(-)-histidine monohydrochloride monohydrate). The basal medium was Medium C, Table I (1), with total amino acids 1.5 times the stated concentrations.

† Six replicate tubes at each level of sample.

‡ The average mean deviations from the mean at the different levels were 2.6 per cent (1 day's incubation) and 4.7 per cent (2 days incubation).

TABLE IV

Summary of Assays of Histidine in Amino Acid Test Mixtures with *Lactobacillus fermenti* 36

Amino acid test mixture No.	Level of total amino acids in basal medium	Incubation time	Histidine recovery	
			Individual	Average
		days	per cent	per cent
1	1.5	1	99.8, 95.0, 101.3	
1	2.5	1	101.5, 100.0, 92.0	
1	1.5	2	102.2, 100.5, 96.9, 92.1	
1	2.5	2	100.4, 103.7, 106.1	99.4
2	1.5	1	103.8, 95.6, 99.8	
2	2.5	1	101.9, 98.5, 87.6*	
2	1.5	2	102.0, 103.5, 101.2, 99.5	
2	2.5	2	102.5, 102.2, 104.6, 106.4	101.6
3	1.5	1	98.5, 102.0	
3	2.5	1	102.0, 93.3	
3	1.5	2	99.0, 98.5	
3	2.5	2	100.6, 106.5	100.0

\* Omitted in calculating the average value.

in 6 inch test-tubes possibly may have resulted because of decreased aeration in the smaller tubes. It was observed in other experiments that there was no growth, even of concentrated inocula, in 6 inch tubes which were shaken continuously.

Acid production was essentially the same at a given time and dilution of inocula in media with and without added hydrolysate of *Lactobacillus fermenti* cells. It would appear, therefore, that *Lactobacillus fermenti* cells contain no acid-stable factor essential for the growth of this microorganism which is not present in the basal medium. In view of these experimental

TABLE V  
Results of Assays of Histidine in Casein Hydrolysate\*

Casein (corrected for moisture and ash) per tube	1 day incubation time				2 days incubation time			
	Titration volume of 0.0310 N NaOH per tube		Histidine found†		Titration volume of 0.0310 N NaOH per tube		Histidine found†	
	Range‡	Average	Per tube	Casein	Range‡	Average	Per tube	Casein
$\gamma$	ml.	ml.	$\gamma$	per cent	ml.	ml.	$\gamma$	per cent
149.2	2.00-2.20	2.03	4.30	2.88	2.82-2.92	2.88	4.40	2.94
298.4	2.80-3.02	2.93	8.72	2.92	4.28-4.90	4.53	9.15	3.06
447.6	3.50-3.83	3.66	12.80	2.86	5.71-6.31	5.95	13.50	3.02
596.8	4.02-4.38	4.24	16.88	2.92	7.38-8.03	7.65	19.38	3.24
746.0	4.50-4.92	4.77	22.36	2.99	8.51-9.58	9.12	24.15	3.23
Average.	....	....	2.91					3.10

\* *Lactobacillus fermenti* 36 and the basal medium (Medium C, Table I (1)) with total amino acids 1.5 times the stated concentrations were employed.

† Six replicate tubes at each level of sample.

‡ The average mean deviations from the mean at the different levels were 1.3 per cent (1 day's incubation) and 3.5 per cent (2 days incubation).

results, it was considered probable that inocula diluted more than 1:5 would not be satisfactory for assay purposes.

The casein, the silk fibroin, and the hydrolysis procedure were the same as those described earlier (7). The experimental data are given in Tables I to IX.

### Histidine in Casein

Evidence has been presented that histidine may be determined with reasonable accuracy in amino acid test mixtures (Tables I to IV) and in casein hydrolysates (Tables V to VII) with *Lactobacillus fermenti*, the basal medium (Medium C, Table I, described in the foregoing paper (1)), and the experimental conditions stipulated in this paper. Although histidine

could not be determined accurately with a basal medium containing total amino acids 1 times the concentrations given previously (1), the results were satisfactory with basal media containing total amino acids 1.5 and 2.5 times these concentrations. The results were unsatisfactory, also, when

TABLE VI  
*Recovery of Histidine Added to Casein Hydrolysate\**

Casein per tube (corrected for moisture and ash)	l(-)-Histidine							
	1 day incubation time				2 days incubation time			
	In casein per tube† (II)	Added per tube (III)	Found per tube (IV)	Recovery‡ (V)	In casein per tube§ (VI)	Added per tube (VII)	Found per tube (VIII)	Recovery‡ (IX)
(I)	(II)	(III)	(IV)	(V)	(VI)	(VII)	(VIII)	(IX)
$\gamma$	$\gamma$	$\gamma$	$\gamma$	per cent	$\gamma$	$\gamma$	$\gamma$	per cent
74.6	2.16	2.00	4.21	102.5	2.31	2.00	4.14	91.5
149.2	4.33	4.00	8.46	103.2	4.63	4.00	8.55	98.0
223.8	6.49	6.00	12.20	95.2	6.94	6.00	13.05	101.8
298.4	8.65	8.00	16.24	94.9	9.25	8.00	16.70	93.1
373.0	10.82	10.00	19.80	89.8	11.56	10.00	21.90	103.4
Average.....				97.1				97.6

\* The casein hydrolysate sample was prepared to contain 10.0  $\gamma$  of histidine per ml. and 373.0  $\gamma$  of casein (corrected for moisture and ash) per ml. Volumes of this solution varying from 0.20 to 1.0 ml. were taken for the assays. The basal medium was Medium C, Table I (1), with total amino acids 1.5 times the stated concentrations. The assays were made with *Lactobacillus fermenti*.

† Estimated on the basis of the 2.91 per cent of histidine in casein given in Table V.

‡ Calculated from the relation,  $((IV - II)/III) \times 100 =$  per cent recovery. The recoveries of histidine in comparable experiments with total amino acids in the basal medium (Medium C, Table I (1)) 2.5 times the stated concentrations were 95.8 and 93.5 per cent.

§ Estimated on the basis of the 3.10 per cent of histidine in casein given in Table V.

|| Calculated from the relation,  $((VIII - VI)/VII) \times 100 =$  per cent recovery. The recoveries of histidine in comparable experiments with total amino acids in the basal medium (Medium C, Table I (1)) 2.5 times the stated concentrations were 99.7, 95.4, and 103.2 per cent.

the incubation period was longer than 2 days. The preferred assay conditions are considered to be a 2 day incubation time and total amino acids 1.5 times the indicated concentrations. Incubation times as short as 1 or 2 days are advantageous in the routine determination of amino acids by microbiological procedures.

It has been found (Table VII) by assay with *Lactobacillus fermenti* that casein, corrected for moisture and ash, contained  $3.0 \pm 0.1$  per cent of histidine. It seems probable that this value is nearly correct, since it was obtained in nine separate assays with *Lactobacillus fermenti* and twelve

separate assays with *Leuconostoc mesenteroides* with two casein preparations, two levels of total amino acids in the basal media, and incubation times of 1 and 2 days with the first organism and 3 to 7 days incubation time with the second. The assay data obtained with *Leuconostoc mesenteroides*

TABLE VII

Summary of Percentages of Histidine in Two Samples of Casein Determined under Different Conditions with Two Microorganisms\*

Organism	Total amino acid level in basal medium	Incubation time	Histidine	
			Individual	Average
		days	per cent	per cent
<i>Lactobacillus fermenti</i> 36	1.5	1	2.90,† 2.96†	
	1.5	1	2.90	
	1.5	2	3.10,† 3.04†	
	1.5	2	3.05	
	2.5	2	3.03,† 3.06†	
	2.5	2	3.07	3.0
<i>Leuconostoc mesenteroides</i> P-60	1	3	3.01	
	1	4	2.94	
	1	5	3.03, 3.05, 3.04, 2.87	
	2	4	3.05	
	2	5	3.04, 3.09	
	1	6	2.88	
	2	6	3.09	
	1	7	2.72†	
	2	7	2.94	3.0

\* Medium C, Table I (1), was employed with *Lactobacillus fermenti* and Medium D, Table I (9), was employed with *Leuconostoc mesenteroides*. The standard histidine was run at fifteen levels, the casein hydrolysate was run at five levels, and six tubes were employed at each level of standard and sample in the experiments with *Leuconostoc mesenteroides*. The histidine values are corrected for the 0.21 per cent moisture and 0.55 per cent ash in the authors' casein and for the 8.57 per cent moisture and 0.93 per cent ash in the S. M. A. casein.

† Values for authors' casein. All other values are for S. M. A. casein.

‡ Omitted in calculating the average value.

probably were highly reliable, since average recoveries (Table VIII) of 100.7, 100.0, and 102.9 per cent were calculated from thirty-two separate experiments on the recovery of histidine from three amino acid test mixtures. It was reported previously (2) that casein contained 3.1 per cent of histidine, corrected for moisture and ash.

#### Histidine in Silk Fibroin

The histidine content of silk fibroin determined with *Lactobacillus fermenti* under the experimental conditions given in Table IX was found to



be 0.33 to 0.35 per cent, corrected for moisture and ash. Although these figures are in close agreement with those reported previously (2), it seems

TABLE VIII

*Recovery of Histidine from Amino Acid Test Mixtures in Experiments with Leuconostoc mesenteroides P-60\**

Amino acid test mixture No.	Total amino acid level in basal medium	Incubation time	Histidine recovery	
			Individual	Average
		days	per cent	per cent
1	1	3	99.3	
	1	4	100.4	
	1	5	103.9, 102.6, 98.7	
	1	6	99.8	
	1	7	96.8	
	2	3	101.3	
	2	4	102.6	
	2	5	104.8, 100.7	
	2	6	100.7	
	2	7	98.5	100.7
2	1	3	100.2	
	1	4	99.8	
	1	5	99.0	
	1	6	98.7	
	1	7	98.2	
	2	3	103.8	
	2	4	98.1	
	2	5	101.4	
	2	6	102.0	
	2	7	98.0	100.0
3	1	3	102.5	
	1	4	101.1	
	1	5	101.9	
	1	6	104.1	
	1	7	103.2	
	2	3	103.1†	
	2	4	103.5	
	2	5	101.6	
	2	6	104.3	
	2	7	103.5	102.9

\* The standard histidine was run at fifteen levels, the amino acid test mixtures were run at five levels, and six tubes were employed at each level of standard and sample. Test Mixture 3 contained 0.0446 per cent histidine.

† Omitted in calculating the average value.

probable that they may be somewhat high, since recoveries of histidine added to silk fibroin hydrolysate were variable and, on the average, about 7 per cent higher than the theoretical amount. It has been emphasized,

previously, that it is difficult to determine accurately amino acids which are present in low proportions in silk fibroin because of the high percentage of glycine, alanine, serine, and tyrosine in this protein.

TABLE IX  
Results of Assays of Histidine in Silk Fibroin Hydrolysate\*

Silk fibroin (corrected for moisture and ash) per tube	1 day incubation time					2 days incubation time				
	Titration volume of 0.0310 N NaOH per tube		Histidine found			Titration volume of 0.0310 N NaOH per tube		Histidine found		
	Range†	Average	Per tube	Silk fibroin		Range†	Average	Per tube	Silk fibroin	
$\gamma$	ml.	ml.	$\gamma$	per cent		ml.	ml.	$\gamma$	per cent	
753	1.71-1.90	1.79	2.78	0.37		2.19-2.22	2.21	2.50	0.33	
1505	2.11-2.31	2.22	5.03	0.34		2.84-3.10	3.03	4.80	0.33	
2258	2.60-2.71	2.66	7.30	0.33		3.84-4.24	3.95	7.50	0.33	
3010	3.11-3.28	3.19	10.11	0.34		4.70-5.33	4.86	10.10	0.33	
3763	3.59-3.79	3.68	12.92	0.34		5.51-6.59	6.29	14.50	0.39	
Average.	.....	.	.	0.34					0.34	

\* *Lactobacillus fermenti* and the basal medium (Medium C, Table I (1)) with total amino acids 1.5 times the stated concentrations were employed. The average mean deviations from the mean at the different levels were 3.4 per cent (1 day's incubation) and 5.4 per cent (2 days incubation). With total amino acids 2.5 times the concentrations stated in Medium C, Table I (1), the corrected values 0.34 and 0.34 per cent (1 day's incubation) and 0.33 and 0.35 per cent (2 days incubation) were found. In recovery experiments with total amino acids in the basal medium  $\times 1.5$ , the recoveries of histidine were 111 and 109 per cent (1 day's incubation) and 110 and 106 (2 days incubation). In comparable experiments with total amino acids  $\times 2.5$ , the recoveries of histidine were 109 and 83 (1 day's incubation) and 103 and 121 (2 days incubation).

† Five or six replicate tubes at each level of sample.

#### SUMMARY

A satisfactory procedure for the determination of histidine in protein hydrolysates with *Lactobacillus fermenti* 36 has been described. The preferred assay conditions were considered to be a 2 day incubation time, inocula dilutions of 1:5, and total amino acids in the basal medium 1.5 times the concentrations stipulated in Medium C, Table I (1).

It has been found by assay of two samples of casein with *Lactobacillus fermenti* 36 and *Leuconostoc mesenteroides* P-60 under varied experimental conditions that casein contains  $3.0 \pm 0.1$  per cent of histidine corrected for moisture and ash. It seems probable, therefore, that  $3.0 \pm 0.1$  per cent is the true value for the percentage of histidine in casein. The figure 3.1 per cent was reported in an earlier paper (2). It is recognized, however,

that the extent to which histidine is destroyed or altered during the hydrolysis of casein must be measured before the true value of histidine in this protein can be determined with certainty. It is considered probable, also, that samples of casein derived from different natural sources and prepared by different methods may not contain identical proportions of amino acids.

Silk fibroin was found to contain 0.33 to 0.35 per cent of histidine corrected for moisture and ash by assay with *Lactobacillus fermenti* 36 under varied experimental conditions. Although the corrected value, 0.34 per cent, was found previously (2) by assay with *Leuconostoc mesenteroides* P-60, it appears probable from experiments on the recovery of histidine from silk fibroin hydrolysates that these values may be higher than the true figure.

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# THE DETERMINATION OF CREATINE AND CREATININE

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In conjunction with the amino acid studies upon human subjects carried out in this laboratory, the urinary creatine and creatinine excretions were measured. These determinations were performed by the method of Folin (1), which was found to be quite satisfactory. Consequently, we were somewhat surprised by the report of Albanese and Wangerin (2) that considerable quantities of urinary creatine are not detectable by the Folin method. They state that part of the creatinine is destroyed by the conditions used to convert creatine to creatinine and describe a modification of the method to correct for this destruction.

In view of the results of Albanese and Wangerin a more careful study of the Folin procedure was undertaken. The experiments reported here do not confirm the claim that in the Folin determination of creatine considerable destruction of creatinine occurs. Certain serious defects of the Albanese modification are also described.

## EXPERIMENTAL

*Creatinine Determination*—The method of Folin (1) was adapted to the Evelyn photoelectric colorimeter with the 520 m $\mu$  filter. The picric acid was purified through sodium picrate as described by Benedict (3), and was tested for purity by the Folin and Doisy method (4). The saturated solution of picric acid recommended by Folin was replaced by a 1.175 per cent solution, as was suggested by Peters (5).

Creatinine was purified by conversion to the zinc chloride salt and subsequent regeneration by treatment with ammonia (6).

$C_4H_7N_3O$ . Calculated, C 42.48, H 6.19; found, C 42.51, H 6.16

A standard solution was prepared by dissolving 1 gm. of creatinine in 1 liter of 0.1 N HCl. In certain experiments in which the HCl was replaced by water a fresh standard solution was prepared each day to eliminate the possibility of decomposition.

A modification of time factors in the determination was made as a result of a preliminary study. In the Folin procedure the mixture is diluted to 100 ml. 10 minutes after the addition of the sodium hydroxide and picric acid solutions and then read immediately. In the concentrations adapt-

able to the Evelyn photoelectric colorimeter the color is completely developed at the end of 3 minutes. To allow a factor of safety we dilute the mixture to volume 6 minutes after the addition of the sodium hydroxide and picric acid solutions. The color of the diluted solution fades slightly for approximately 16 minutes and then remains stable for several hours. This fading occurs irrespective of the length of time that the color is allowed to develop before dilution. It has been found convenient to determine the color intensity 20 minutes after the dilution to volume.

TABLE I

*Decomposition of Creatinine by Autoclaving with Picric Acid at 121°*

The indicated quantity of creatinine dissolved in 1 ml. of either water or 0.1 N HCl was autoclaved with 20 ml. of picric acid solution.

Autoclaving time	Solvent for creatinine	No. of determinations	Creatinine added	Creatinine recovered, range	Creatinine decomposition, average
min.			mg.	mg.	per cent
20	Water	6	0.200	0.196-0.199	0.8
20	0.1 N HCl	11	0.200	0.197-0.200	0.7
20	0.1 " "	6	0.500	0.495-0.502	0.4
40	Water	6	0.200	0.198-0.199	0.7
40	0.1 N HCl	6	0.100	0.095-0.101	1.0
40	0.1 " "	6	0.200	0.196-0.199	1.2
40	0.1 " "	11	0.500	0.493-0.502	1.0
60	Water	5	0.200	0.190-0.196	3.6
60	0.1 N HCl	6	0.200	0.190-0.193	4.1
80	Water	6	0.200	0.192-0.196	3.0

*Creatine Determination*—Creatine was purified by recrystallizing from water and drying at 120° for 24 hours.

$C_4H_7N_3O_2$ . Calculated, C 36.64, H 6.87; found, C 36.89, H 6.92

A standard solution was prepared by dissolving 1 gm. of creatine in 1 liter of water. This was freshly prepared each day to avoid decomposition.

*Decomposition of Creatinine*—A study was first made of the stability of creatinine under the experimental conditions used for the conversion of creatine to creatinine. To a measured quantity of creatinine were added 20 ml. of picric acid solution and the mixture was then treated by either the autoclave or boiling method of Folin (1). Approximately 2 hours on a hot-plate were required in the boiling method to attain the original volume. Varying periods of time were used in the autoclave procedure as shown in Tables I to IV. The amount of creatinine decomposition was then determined by colorimetric measurement.

In twelve repetitions of the experiment in which the boiling method was employed, 96 to 99 per cent of the creatinine was recovered unchanged, with an average recovery of 98 per cent. In Table I is shown the creatinine decomposition that occurred under the conditions used for the determination of creatine by the autoclave procedure. The experiments in which water served as solvent in the standard creatinine solutions are more comparable to a urinary creatine determination than are those in which 0.1 N HCl was the solvent. The latter were included for a comparison between our results and those of Albanese. In all of the present experiments the creatinine decomposition was slight and never more than one-half of the 9 per cent value reported by Albanese and Wangerin. It would appear from these results that the small destruction of creatinine in the Folin creatine determination is not a serious error in the method.

TABLE II

*Decomposition of Creatinine by Autoclaving at 121° without Picric Acid*

0.2 mg. of creatinine dissolved in 1 ml. of either water or 0.1 N HCl was autoclaved.

Autoclaving time	Solvent for creatinine	No. of determinations	Creatinine recovered, range	Creatinine decomposition, average
min.			mg.	per cent
20	0.1 N HCl	6	0.192-0.199	2.8
20	Water	6	0.163-0.170	16.6
40	0.1 N HCl	11	0.187-0.192	5.5
40	Water	6	0.152-0.158	22.2
60	0.1 N HCl	6	0.184-0.192	6.1
80	0.1 " "	5	0.182-0.187	7.6

In the Albanese modification to correct for the creatinine decomposition, the creatine is determined as the difference between the total creatinine following autoclaving with picric acid and the preformed creatinine following autoclaving without picric acid. This method is applicable only if the quantity of creatinine destroyed by autoclaving is the same irrespective of the presence or absence of picric acid. A comparison between Table I and Table II indicates that this is not the case.

When an aqueous solution of creatinine was autoclaved without picric acid, considerable quantities disappeared. The presence of 0.1 N HCl aided in suppressing this decomposition but picric acid was even more effective in this respect. The HCl solutions used in the tests summarized in Table II showed pH values of 1.0. Albanese and Wangerin (2) added HCl to urine samples with a resultant pH of 4 to 5 before diluting to a standard volume. Since the extent of creatinine destruction by autoclaving without picric acid is dependent upon the pH, it is probable that a

greater destruction will be obtained when urine samples of pH 4 to 5 are so autoclaved than when 0.1 N HCl solutions are autoclaved. In any event, considerable destruction of creatinine occurs irrespective of the pH when autoclaving is carried out in the absence of picric acid. Thus the creatinine destruction in the determination of preformed creatinine and the very slight destruction in the determination of total creatinine result in creatine values which are too high. Incidentally, if urine is preserved by

TABLE III  
*Conversion of Creatine to Creatinine*

1 ml. of an aqueous solution containing 0.2 mg. of creatine was treated with 20 ml. of picric acid solution. The theoretical amount of creatinine to be recovered is 0.173 mg.

Method	No. of determinations	Autoclaving time	Actual creatinine recovered, range	Conversion average
		min.	mg.	per cent
Boiling.....	17		0.167-0.176	98.4
Autoclave .....	20	20	0.113-0.134	70.8
" .....	12	40	0.155-0.159	91.3
" .....	12	60	0.164-0.167	95.1
" .....	17	80	0.167-0.171	98.0
" .....	6	100	0.167-0.170	97.6

TABLE IV  
*Determination of Creatine in Mixture of Creatine and Creatinine*

Method	No. of determinations	Creatine present	Creatine present	Theoretical creatinine to be recovered	Actual creatinine recovered, range	Conversion average
		mg.	mg.	mg.	mg.	per cent
Boiling.....	7	0.200	0.200	0.373	0.365-0.370	97.5
" .....	10	0.100	0.100	0.186	0.180-0.189	97.7
80 min., autoclave..	5	0.100	0.100	0.186	0.184-0.185	98.4

toluene in the usual manner without the addition of HCl, the Albanese and Wangerin modification gives extremely high creatine values. It must be concluded that the Folin method is much more accurate than the Albanese modification.

*Conversion of Creatine to Creatinine*—The efficiency of the conversion of creatine to creatinine was studied by subjecting standard aqueous creatine solutions to either the Folin boiling or autoclave method (1). The data are presented in Table III.

The boiling procedure gave an average conversion of 98.4 per cent, which is considerably better than the 86 per cent reported by Albanese and Wan-

gerin (2). In the case of the autoclave method the extent of conversion increased with increasing autoclaving time until a maximum of 98 per cent was attained at 80 minutes. This compares with the 93 per cent value obtained by Albanese at the end of 60 minutes.

The creatine conversion was also studied by adding a standard solution of creatine to a standard solution of creatinine and treating the resultant mixture by the boiling or 80 minute autoclaving procedure. The results are shown in Table IV. Again either method produced a 97 to 98 per cent conversion. It would appear that both procedures give excellent results in the determination of creatine.

#### SUMMARY

1. A study was made of the Folin method for the determination of creatine. The report that considerable quantities of creatinine are destroyed by the conditions used for the conversion of creatine to creatinine was not confirmed.

2. Creatinine is destroyed only slightly when autoclaved with picric acid but to a considerable extent when autoclaved without picric acid. Therefore, the Albanese and Wangerin modification yields creatine values that are too high.

3. Either the boiling or autoclave method of Folin produces a 97 to 98 per cent conversion of creatine to creatinine. For the autoclave procedure the optimum conversion occurs in 80 minutes at 121°.

4. A slight modification of time factors is described for adapting the creatine and creatinine determinations to the Evelyn photoelectric colorimeter.

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# THE DETERMINATION OF CATECHOL, PHENOL, AND HYDROQUINONE IN URINE

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There have been many methods devised for the determination of urinary phenols based on colorimetric, gravimetric, and titrimetric procedures, but few of them include quantitative determinations of hydroquinone and catechol. These two phenols are produced in considerable amounts in animals exposed to benzene (1) and since we wished to study this problem further a convenient method of analysis had to be devised.

The present paper describes a relatively simple method for the determination of catechol, phenol, and hydroquinone in urine. All titrations are made with a single solution and only one primary standard, potassium bromate, is required.

## Reagents—

2 M pyridine-acetate buffer. 160 ml. of pyridine and 10 ml. of glacial acetic acid diluted to 1 liter; pH 6.5.

Lead acetate solution, 37.9 gm. of  $\text{Pb}(\text{CH}_3\text{COO})_2 \cdot 3\text{H}_2\text{O}$  per liter. Add 1 ml. of glacial acetic acid.

Potassium iodate solution, saturated.

Sodium hydroxide solution, 30 per cent.

0.2 N bromate-bromide solution. 5.568 gm. of  $\text{KBrO}_3$  and 20 gm. of  $\text{KBr}$  per liter.

0.2 N sodium sulfite solution. 12.6 gm. of anhydrous  $\text{Na}_2\text{SO}_3$  per liter protected from air by  $\text{CO}_2$  and standardized frequently against the bromate solution.

Ethyl alcohol, 95 per cent.

Ether, washed with sodium hydroxide-potassium permanganate solution until alcohol and aldehyde are absent, and then distilled.

Sodium bicarbonate, 10 grain tablets and powder.

*Hydrolysis of Phenolic Esters*—Hydrolysis of the esters is carried out in the extraction tube in the inverted position, A, Fig. 1.

Measure 25 ml. of centrifuged urine<sup>1</sup> into an extraction tube and add 8 drops of concentrated sulfuric acid. Insert a rubber stopper and tip the tube in a horizontal position with the side arm up until the  $\text{CO}_2$  has

<sup>1</sup> With acid urines containing no precipitated carbonates no centrifuging is required.

escaped. The pH is now about 1.0, which is adequate for hydrolysis of the esters in 2 hours at  $100^{\circ}$ .

The adapter is connected to the side arm and to the condenser with the *extraction tube* immersed in boiling water.

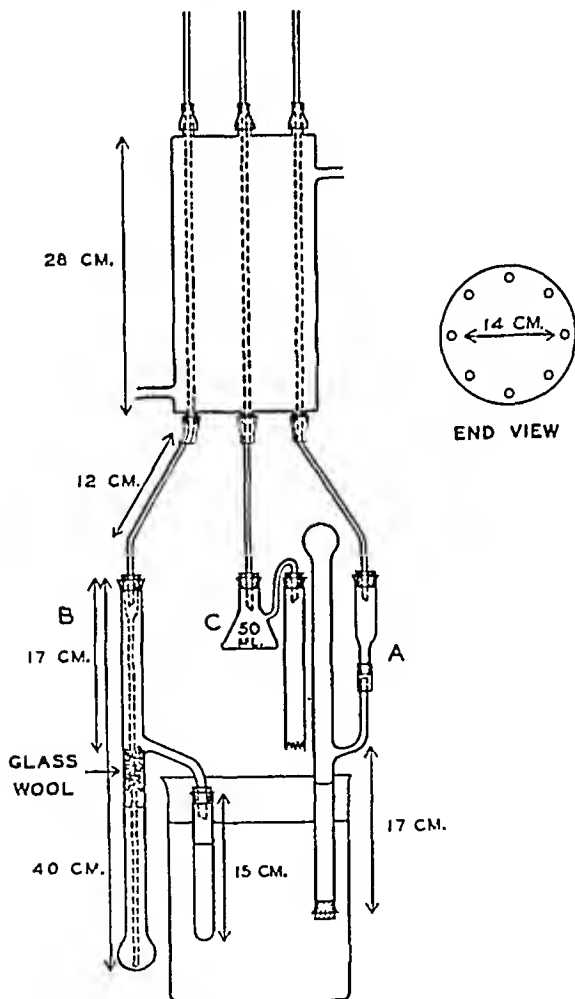


FIG. 1. Apparatus for the hydrolysis and extraction of urine and concentration of the extract. The letters represent the respective positions.

*Extraction of Phenols at pH 7.0*—After hydrolysis of the esters at pH 1.0, the extraction tube is removed from the bath and the urine is saturated with sodium sulfite. This neutralizes sulfuric and phenolic acids and brings the pH up to 7. The mono- and diphenols may, however, be quan-

titatively extracted and the extract does not need further washing. Sulfite has the advantage over bicarbonate in furnishing  $\text{SO}_2$  for reduction of any quinones which may have formed.

The urine is cooled and the apparatus is assembled for extraction as in *B*, Fig. 1. The glass wool plug on the funnel tube aids in the separation of ether from the urine. A few small pieces of pumice (40 mesh) are added to the ether boiler to prevent bumping. The water bath is kept at  $55^\circ$  and extraction is continued for 4 hours.

The rate of extraction may be considerably increased by wrapping a strip of lead (not shown) in a spiral around the lower third of the funnel tube. This facilitates saturation of each drop of ether which passes through the urine. By this means extraction is complete in about 3 hours, provided the rate of ether vaporization is maximal. This can be assured by keeping the boiling tubes filled about two-thirds full of ether. It has been shown that the rate of ether vaporization in the long narrow tubes with pumice present is dependent on the height of the ether column. This follows from the fact that the whole column is full of bubbles and therefore the effective surface for evaporation of ether is greatly in excess of the cross-sectional area of the tube.

*Concentration of Extract and Collection of Ether*—When extraction is completed, the extract is concentrated to dryness by inserting the collection flask as shown in position *C*, Fig. 1, first with the funnel tube in place and second with the funnel tube removed. Water is added to the urine to displace nearly all the ether from the extraction tube into the ether boiler. Concentration of the extract is then completed.

The ether may be used over again without further treatment.

### *Analysis of Extract*

*Separation of Catechol*—After extraction and concentration of the extract, catechol is separated as the lead salt at pH 6.5.

A battery of small sintered glass funnels, of medium porosity,<sup>2</sup> mounted for suction filtration is used for the separations to follow.

About 2.0 ml. of 0.1 *M* lead acetate solution are placed in one of the funnels. 3 ml. of 2 *M* pyridine-acetate buffer, pH 6.5, are added to the concentrated extract in the ether boiler and this is rinsed into the funnel with a few ml. of water. The mixture is stirred and catechol is immediately and quantitatively precipitated as a lead salt. After a minute it settles out, and is filtered with suction. The filtrate containing phenol and hydroquinone is received in a 100 ml. wide mouth, screw cap bottle.<sup>3</sup> The

<sup>2</sup> Pyrex, catalogue No. 33730.

<sup>3</sup> A. H. Thomas 4 ounce bottle, No. 6284; plastic caps, No. 2849. Remove the cork liner and pour in a layer of paraffin to protect the cap from bromine fumes.

precipitate is washed free of soluble lead salt with a minimum of water (about 12 ml.) which is added to the filtrate and treated as described in the following section.

The precipitate of lead catecholate is dissolved with 2 ml. of glacial acetic acid and washed into a 150 ml. beaker with water. An excess of saturated potassium iodate solution is added and the mixture heated on a hot-plate until lead iodate settles out. (Catechol is oxidized by iodate to a soluble red substance the color of which gives a useful index of the amount of catechol present.)

Lead iodate is filtered cold and washed, the filtrate discarded, and the original beaker in which precipitation occurred is returned as the receiver. Lead iodate is dissolved with 2 ml. of 30 per cent sodium hydroxide and washed into the beaker.<sup>4</sup> 1.0 ml. of concentrated sulfuric acid and 0.5 gm. of potassium iodide are added, and the liberated iodine titrated with 0.2 N sodium sulfite solution, a platinum-platinum polarized electrode being used as indicator.

While it is true that thiosulfate and starch indicator may be used in this titration, sulfite must be used in the phenol determination and the electrode must be used in the hydroquinone determination. All three titrations may be made with sulfite and the electrode indicator.

Sulfite must be protected from oxidation by the air, preferably by storing it under an inert gas such as carbon dioxide. When properly protected the solution is stable indefinitely. We have found the following arrangement perfectly satisfactory: A 5.0 ml. micro burette graduated in hundredths of a ml. is fitted with a 3-way stop-cock and is filled by siphon from a 4 liter stock bottle. The latter is connected to a Kipp generator for CO<sub>2</sub> and the top of the burette is connected to the CO<sub>2</sub> space by means of a glass tube. Short rubber connections must be used throughout.

*Calculation—*

$$\text{Mg. catechol} = \frac{(S - B) \times N \times 110.1}{12}$$

$S$  = ml. of sulfite used for the sample,  $B$  = ml. of sulfite used for the blank,  $N$  = the normality of the sulfite.

The blank for benzene-exposed animals is a sample of urine taken before exposure and treated exactly as the sample after exposure is treated.

For other purposes 25 ml. of water may be substituted for urine. These blanks are usually very small and for some purposes entirely negligible.

*Determination of Phenol—*The filtrate from lead catecholate is acidified with 1.0 ml. of concentrated sulfuric acid and 5.0 ml. of 0.2 N bromate-bromide mixture are added. This is sufficient to brominate about 8.0 mg.

<sup>4</sup> Before the funnels are used again they must be washed with acid and water.

of phenol under these conditions. A small vial containing about 1.0 gm. of potassium iodide is placed in the bottle and the cap is screwed on.<sup>5</sup>

After about 30 minutes, when bromination is complete, the vial is tipped over and the excess bromine liberates an equivalent quantity of iodine from the iodide. The bottle is opened and the vial removed and washed carefully with alcohol, the washings being added to the mixture in the bottle. Alcohol is used in order to bring any precipitated iodine into solution and to prevent the precipitation of oxidized hydroquinone in the next step of the analysis.

The iodine is titrated with 0.2 *N* sulfite solution, the electrode being used as indicator.

*Calculation—*

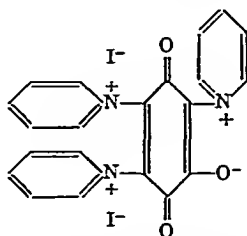
$$\text{Mg. phenol} = \frac{(B - S) \times N \times 94.11}{6}$$

*B*, *S*, and *N* have the same significance as the corresponding symbols in the catechol determination.

It will be observed that this procedure is an adaptation of the Koppeschaar method (2) for phenol. The change from thiosulfate to sulfite was necessary because hydroquinone is to be determined in the same mixture.

It was shown that tetrathionate resulting from the oxidation of thiosulfate was further oxidized by iodine after the acid was neutralized by bicarbonate. Sulfite, on the other hand, is oxidized to sulfate, which is stable in bicarbonate buffer.

*Determination of Hydroquinone*—In our early experiments we used acetate buffer to control pH in the catechol precipitation. The buffer



1,1',1''-(2,5-Dihydro-3-hydroxy-2,5-dioxo-*p*-phenylene)-  
tripyridinium betaine diiodide

<sup>5</sup> A convenient indication that an excess of bromine is present is the color of the potassium iodide in the vial. If it is brown on top, sufficient bromate was added. If after about 10 minutes the potassium iodide is not brown, the bottle may be opened and another 5.0 ml. of bromate solution added. When only 0.5 ml. of sulfuric acid was used, the bromine uptake was too high and the subsequent hydroquinone recovery was low.

appeared with phenol and hydroquinone in the filtrate. Under these conditions hydroquinone consumed 2 equivalents of iodine per mole and quinone was formed.

When pyridine was substituted for acetate, hydroquinone consumed 10 equivalents of iodine per mole and a new compound was formed having the probable structure (3) shown in the accompanying diagram.

The procedure is as follows: The mixture from the phenol determination still contains unchanged hydroquinone. Add 5.0 ml. of potassium bromate-bromide solution which liberates an excess of *iodine*, since KI is also present at this point. The bottle is covered with a watch-glass and the sulfuric acid is neutralized by adding three sodium bicarbonate tablets, one at a time. It was found impractical to use the powder at first because of the spraying resulting from the rapid release of carbon dioxide. After the tablets have dissolved, an excess of powdered sodium bicarbonate is added.

TABLE I  
*Recovery of Phenols*

Amount of each in mixture	Found without extraction			Found with extraction from urine		
	Catechol	Phenol	Hydroquinone	Catechol	Phenol	Hydroquinone
mg.	mg.	mg.	mg.	mg.	mg.	mg.
2.2	2.15	2.19	2.22	2.24	2.23	2.22
4.4	4.44	4.36	4.47	4.36	4.17	4.32
6.6	6.62	6.30	6.54	6.14	6.26	6.71
8.8	8.96	8.48	8.72	8.79	8.87	8.69

The watch-glass is rinsed with alcohol and the mixture allowed to stand for 1 hour, after which it is titrated with sulfite.

During the oxidation of hydroquinone a red precipitate sometimes appears. This must be brought back into solution by the addition of a few ml. of alcohol. If it is allowed to remain separated, the oxidation will be incomplete and the end-point in the titration of excess iodine will be uncertain, since the precipitate carries down free iodine.

*Calculation—*

$$\text{Mg. hydroquinone} = \frac{(B - S) \times N \times 110.1}{10}$$

*B*, *S*, and *N* have same significance as above.

A few typical analyses are given in Table I. The first recoveries were made by analysis of a mixture of standard solutions of the three phenols without extraction; the second recoveries were made by adding the three standard solutions to 25 ml. of normal human urine and analyzing the mixture as described. A urine blank was also analyzed and the values found were subtracted from the totals to give those in Table I.

Fig. 2 is a typical set of curves showing the excretion of phenol, hydroquinone, and catechol by a rabbit during a series of injections of benzene in olive oil.

It will be observed that all curves are roughly parallel, which suggests a dependence of the secondary products of oxidation (hydroquinone and catechol) on the concentration of the primary product (phenol).

The peak occurred at the 9th day and, in spite of continued injections, the amount of urinary phenols declined sharply and the rabbit died on the 17th day.

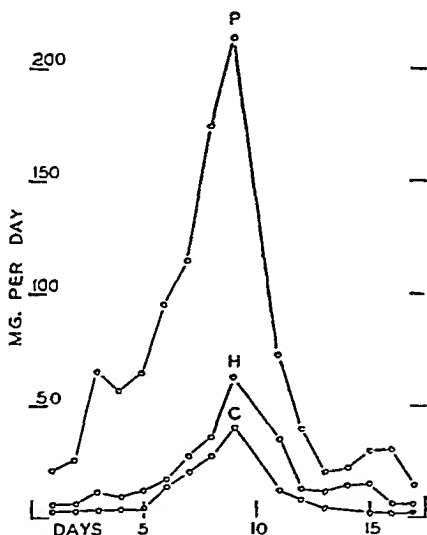


FIG. 2. Excretion of phenol (Curve P), hydroquinone (Curve H), and catechol (Curve C) by a rabbit during the daily subcutaneous injection of 2 ml. of benzene in 2 ml. of olive oil.

The count of white blood cells declined from 11,000 on the 2nd day to less than 1000 on the 17th.

Further studies are in progress.

#### DISCUSSION

The specificity of these reactions is obviously not absolute. It is especially important that no trace of urine be allowed to pass into the ether boiler and with certain urines this has been a difficult problem. Contamination with urine is easily discovered during the precipitation of catechol, for a heavy rather insoluble precipitate of lead sulfate, phosphate,



and phenolic acids is produced. These determinations must be discarded. "Phenol" determinations on such urines are also high. Acetone and other ketones will react like hydroquinone.

Our "phenol" fraction contains *p*-cresol and other substances such as alcohols, which can reduce bromine.

In spite of these interferences our results indicate that for urine of rabbits exposed to benzene, at least, the interpretations are usually uncomplicated and suitable controls can be devised.

#### SUMMARY

1. A system of analysis of urine for catechol, phenol, and hydroquinone has been described.

2. The extraction of phenols from urine has been improved by the use of sodium sulfite buffer which keeps diphenols reduced and holds back ether-soluble acids.

3. The precipitation of lead catecholate from the ether extract is controlled at pH 6.5 by the use of pyridine-acetate buffer and the separation of the lead as iodate instead of the usual chromate yields more iodine per atom of lead.

4. It has been shown that phenol may be brominated in the presence of hydroquinone, provided sufficient acid is present.

5. A new oxidation of hydroquinone is utilized in which 10 atoms of iodine are reduced per mole, resulting in the formation of a tripyridinium quinone betaine.

6. The methods are simple and fairly specific when the separations are successful. They have been applied to the analysis of rabbit urine during exposure to benzene and a typical set of curves is given.

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# CONSTITUENTS OF THE BLOOD OF THE HIBERNATING AND NORMAL RATTLESNAKE, *CROTALUS HORRIDUS*

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Studies of the blood chemistry of hibernating animals have not been numerous but they have been made on both carnivorous and herbivorous animals.

Vierordt (1) in 1854 found that in the European marmot the red blood corpuscles dropped from 5,800,000 per c.mm. at the beginning of a winter sleep to 2,300,000 at the end of hibernation. Valetin (2) and Quincke (3) in 1881 confirmed Vierordt's findings. Quincke (3) found 64 per cent of the normal amount of hemoglobin in the hibernating marmot. Dubois (4) in 1896 confirmed the studies of the above authors and attributed the decrease in the red cells to an actual destruction of these cells.

In 1916, Rasmussen (5) found that there was about a 20 per cent decrease in red cells and hemoglobin after the hibernating American woodchuck had eaten and had drunk.

Luck and Keeler (6) reported on the blood chemistry of two species of rattlesnakes, *Crotalus atrox* and *Crotalus oregonus*. None of their animals would eat and so they resorted to forced feeding of the former species; and since the latter were small and abundant locally, they bled each animal only once and then within 3 days to 4 weeks of capture. A period of at least 5 days was allowed to elapse after feeding before sampling, and no animal was bled more than once a week.

We studied the blood chemistry of the same snake (*Crotalus horridus*, timber rattler) during a winter fast and then again after several weeks of feeding. The animal was caught near Tuscaloosa, Alabama; its diet has consisted of small rodents, birds, and water. It usually eats periodically from early spring up to and including August. However, in 1942, it did not eat after July. In 1944, the animal took food the last time on August 28. It became sluggish (hibernating) early in the fall, although it was kept in the laboratory which had a temperature of about 24° during the daytime and was occasionally as low as 4.4° during some nights. The animal became active in March, 1945, and shed its skin on March 30, 1945. The snake was 44 inches long and weighed about 1700 gm.

## EXPERIMENTAL

We took a 27.7 cc. sample of heart blood on April 7. The following determinations were made on this blood, which was from the hibernating

animal: non-protein N (7), urea N (8), amino acid N (9), uric acid (10), creatine (8), creatinine (8), chlorides (11), inorganic phosphorus (12), lipid phosphorus (13), cholesterol (14), reducing sugar (15), calcium (16), magnesium (16), hemoglobin, the number of red blood corpuscles, and the volume of the corpuscles.

TABLE I  
*Composition of Rattlesnake Blood*

	<i>Crotalus atrox</i> (Luck and Keeler)	<i>Crotalus oregonus</i> (Luck and Keeler)	<i>Crotalus horridus</i>	
			Hibernating	Normal (active)
	mg. per cent	mg. per cent	mg. per cent	mg. per cent
Non-protein N..	23.4, 20.5, 18.8, 25.3		33.71*	35.99*
Urea N.....	0, 0-1.7, 0.5-1.5, 0-0.7	0	5.47*	11.36*
Amino acid N..	14.7, 17.9, 15.9, 15.8	21.3, 25.9	22.86*	22.18*
Uric acid.....	1.9, 1.2, 1.2, 2.3, 2.6	1.5	1.32*	2.99*
Creatine.....	4.7, 4.8	5.3	2.81*	2.88*
Creatinine.....	1.1, 1.3	1.2	1.13*	1.37*
Chlorides (NaCl)	780, 783, 791		624*	650*
Inorganic P.....	5.2	10.0, 10.7	4.06†	10.61†
				7.13†
Lipoid P.....	19, 14, 11, 17		21.46†	18.25†
Cholesterol.....	99, 101	172	145.76†	149.38†
				241.3†
Reducing sugar..	50, 67, 68, 54	48, 48	60.20*	62.62*
Calcium.....			8.17†	24.80†
Magnesium.....			1.95†	2.4†
Red cells.....			1,520,000†	1,140,000†
Cell volume.....			44.97†	34.17†
Hemoglobin (Sahli) .....			57†	66.2†

\* Folin and Wu protein-free filtrate.

† Serum.

‡ Whole blood.

The snake took the first food on April 13 and by June 18 it had ingested 1464 gm. of food. On July 1 (12 weeks after the first sample and 12 days after the last food was ingested) we took 34.2 cc. of heart blood and the above determinations were repeated.

### Results

The results are presented in the last two columns of Table I. The corresponding values obtained by Luck and Keeler (6) for the first eleven blood constituents are included for comparison.

There was about a 125 per cent increase in uric acid content of the normal

specimen over the hibernating specimen. The inorganic phosphorus of serum was about 75 per cent higher in the normal specimen. The lipid phosphorus was slightly lower in the normal specimen than it was in the blood of the hibernating animal. The serum cholesterol increased about 65 per cent after the animal took food. However, the cholesterol content of the whole blood of the fed animal was of the same order as that of the serum when the animal was hibernating. There was about a 200 per cent increase in calcium and a 23 per cent increase in magnesium.

There is a 25 per cent decrease in the number of red blood cells and a 24 per cent decrease in the volume of cells in the blood following a 12 week feeding period. In spite of the lowered red cell count in the normal specimen, the hemoglobin content rose slightly.

#### DISCUSSION

There seems to be a variation in the blood chemistry of the species of rattlesnakes reported by Luck and Keeler (6) and that of *Crotalus horridus*. However, it is difficult to make a comparative analysis of the results reported by the above authors and our findings, since they used many animals and apparently did not make a complete analysis on a single specimen of blood from either species. Their results only show possible ranges in values for different blood constituents for animals under their special handling conditions. It is interesting to note that some of our results are of the same order as those obtained by Luck and Keeler (6) in their random sampling, while others vary markedly.

The length of the fasts in Luck and Keeler's (6) animals was not controlled.

The urea values by the Folin and Wu method (8) are higher than those reported by Luck and Keeler (6) on other species of rattlesnakes. However, the Folin and Wu method (8) is reported to give high values in the case of birds (17).

The high uric acid value of our normal specimen may be explained by the fact that this substance accounts for the major part of nitrogenous waste in snakes and that the snake's metabolism was at a higher level following the ingestion of food.

The creatine value for the blood of *Crotalus horridus* was somewhat lower than that of either *Crotalus atrox* (6) or *Crotalus oregonus* (6), although the creatinine of all three species was of the same order.

The chlorides showed a small increase in the normal specimen, and in both specimens the values were lower than that for *Crotalus atrox* (6).

The high calcium value in the feeding animal is probably due to the fact that it ingested almost its own weight of whole animals during 9.5 weeks.

The decrease in the number of red blood cells was probably influenced

by the large sample of blood taken. The hemoglobin content was slightly increased in the well fed animal, and since there was a decrease in the number of corpuscles, each cell carried more hemoglobin than it did in the hibernating animal.

#### SUMMARY

1. Blood analyses were made on both hibernating and normal samples of blood from the same rattlesnake, *Crotalus horridus*.

2. There was a marked increase in the following constituents in the normal specimen when compared with the hibernating blood: urea nitrogen, uric acid, inorganic phosphorus, cholesterol, calcium, and magnesium.

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# THE EFFECT OF PHOSPHATE ON THE SOLUBILITY OF CALCIUM CARBONATE AND OF BICARBONATE ON THE SOLUBILITY OF CALCIUM AND MAGNESIUM PHOSPHATES

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In 1925, Holt, La Mer, and Chown (1) reported that the apparent solubility product  $[Ca^{++}]^2[PO_4^{=}]^2$  was greater in a solution of inorganic salts approximating the composition of serum than it was in solutions of sodium chloride of the same ionic strength. Very shortly thereafter, Sendroy and Hastings (2) recalculated values for  $pK'_{Ca_3(PO_4)_2}$  from the data of Holt, La Mer, and Chown and also reported extensive data of their own. According to Sendroy and Hastings, the solubility of calcium phosphate was the same in "serum salt" solutions as in sodium chloride solutions and was between those values calculated from the data of Holt, La Mer, and Chown for the solubility in sodium chloride and in "serum salt" solutions. Sendroy and Hastings suggested (p. 821) that the latter difference might be due to the "use of what may be uncertain values for the first or the third stoichiometric dissociation constant of phosphoric acid at such low  $p\alpha H^+$ ," but they seem to have made no attempt to calculate whether or not any conceivable error in these dissociation constants could have accounted for the observed 100-fold change in the apparent solubility product. We have made such calculations and do not find that any conceivable error in any, or all, of the values for the dissociation constants of phosphoric acid could produce any significantly different effect upon the ratio of  $PO_4^{=}$  to total phosphorus at pH 5.2 or 7.7, which represent the extreme values in the two sets of experiments by Holt, La Mer, and Chown.

The "serum salt" solutions employed by Holt, La Mer, and Chown contained, besides calcium hydroxide and phosphoric acid, sodium, potassium, and magnesium chlorides and sodium bicarbonate. We have since reported evidence for the existence, in solution, of undissociated calcium and magnesium carbonate, bicarbonate, and phosphate (3, 4). However, the amounts of such complexes that could have been present could account for only a small part of the observed greater solubility in "serum salt" compared with that in sodium chloride solutions.

The composition of the "serum salt" solutions used by Sendroy and Hastings is not given. They do not seem to have contained magnesium.

Moreover, it is stated ((2) p. 799) that, in the "sodium chloride" series, the ionic strength up to 0.03 M was furnished by sodium bicarbonate and only above that by sodium chloride. This suggested that the greater solubility in "serum salt" solutions observed by Holt, La Mer, and Chown might have been due to the bicarbonate. If this were the case, no difference in solubility was to be expected in the two series of experiments by Sendroy and Hastings, since both contained bicarbonate.

It was obviously desirable to repeat the work, with sodium chloride- and bicarbonate-containing solutions at approximately the same pH.

TABLE I  
*Solubility of Calcium Phosphate in Sodium Chloride Solutions ( $\mu \approx 0.155$ )*

Experiment No.	pH	Ca <i>moles <math>\times 10^4</math></i>	P <i>moles <math>\times 10^4</math></i>	CaHPO <sub>4</sub> <i>moles <math>\times 10^4</math></i>	$\frac{-\log [Ca^{++}][HPO_4^-]^2}{[aH^+]^2}$
S34*	6.80	710	780	8	2.67
S36*	7.05	477	622	6	2.68
S37*	7.18	423	597	6	2.53
S38*	7.33	292	523	4	2.75
S39*	8.02	106	432	1	2.68
931†	6.95	730	345	5	2.90
932†	7.02	770	330	5	2.68
933†	6.80	784	430	5	3.05
934†	6.70	1135	525	10	2.80
935†	6.68	1300	670	11	2.38

Average, calculated from values for  $[Ca^{++}][HPO_4^-]^2/[aH^+]^2$ .....  $2.678 \pm 0.067$

\* Equilibrated for 29 days.

† Equilibrated for 60 days.

#### EXPERIMENTAL

The general conduct of the experiments and the methods of analysis were those reported in previous work (3-5). The bicarbonate content was determined by adding an excess of standard acid, boiling to expel CO<sub>2</sub>, titrating the excess acid, and correcting for the phosphate present and, in the more alkaline solutions, for the amount of carbonate. Total carbon dioxide was then calculated.

The mixtures were kept at room temperature, about 22°, for the periods indicated in Tables I to IV, and were shaken several times almost every day.

The following constants were used: for H<sub>2</sub>CO<sub>3</sub>, pK<sub>1</sub>' = 6.34 and pK<sub>2</sub>' = 10.10; for H<sub>3</sub>PO<sub>4</sub>, pK<sub>2</sub>' = 6.78; for  $-\log[Ca^{++}][HCO_3^-]/[CaHCO_3^+]$  = 0.81; for  $-\log[Ca^{++}][CO_3^{--}]/[CaCO_3]$  = 3.00; for  $-\log [Ca^{++}][HPO_4^-]/[CaHPO_4]$  = 1.50; and 0.77, 2.31, and 1.50 for the negative logarithms of

the instability constants of the corresponding magnesium complexes. For the calculations from the data of Sendroy and Hastings and for those calculated by them from the data of Holt, La Mer, and Chown, we employed the constants used by Sendroy and Hastings for  $\text{H}_2\text{CO}_3$  and  $\text{H}_2\text{PO}_4$ , the instability constants given above, and  $-\log[\text{Ca}^{++}]^2[\text{HPO}_4^-]^2/[\text{aH}^+]^2 = 4.11$  (5).

TABLE II

*Solubility of Calcium Phosphate and Calcium Carbonate in Solutions Containing Both  $\text{HPO}_4^-$  and  $\text{HCO}_3^-$  ( $\mu \approx 0.155$ ); Equilibrated 20 to 50 days*

Experiment	pH	Total			Calcium as					$-\log \frac{[\text{Ca}^{++}][\text{HPO}_4^-][\text{HCO}_3^-]}{[\text{aH}^+]^2 [\text{Ca}_3(\text{HPO}_4)_2]}$
		Ca	P	$\text{CO}_2$	$\text{CaHCO}_3^+$	$\text{CaCO}_3$	$\text{CaHPO}_4$	$[\text{Ca}_3(\text{PO}_4)_2]$	$[\text{Ca}_3(\text{HPO}_4)_2]$	
		moles $\times 10^4$	moles $\times 10^4$	moles $\times 10^4$	moles $\times 10^4$	moles $\times 10^4$	moles $\times 10^4$	moles $\times 10^4$	moles $\times 10^4$	
A <sub>4</sub>	6.90	1485	1790	258	51	4	9	20	490	1.43
A <sub>2</sub>	7.00	1250	1390	260	51	5	7	19	384	1.31
A <sub>2</sub>	7.15	1180	1360	255	40	5	6	28	292	1.37
A <sub>1</sub>	7.32	848	1190	251	31	7	5	28	267	1.25
A <sub>0</sub>	7.50	676	1090	251	24	7	4	33	207	1.20
B <sub>2</sub>	7.85	504	1010	248	11	9	2	54	150	1.33
B <sub>2</sub>	8.30	274	887	245	4	10	1	58	58	1.37
B <sub>2</sub>	8.63	166	824	256	2	10	0	48	23	1.31
B <sub>4</sub>	8.90	126	800	263	1	10	0	44	11	1.27
B <sub>5</sub>	9.01	120	826	273	1	10	0	44	9	1.27
-Log of average of K... ..										1.33

Initial  $[\text{Ca}] = 0.001 \text{ M}$ ;  $[\text{P}] = 0.00125 \text{ M}$ ;  $[\text{CO}_2] = 0.025 \text{ M}$ . Solid phase as in experiments of Table I.  $[\text{Ca}^{++}]$  in Series A calculated from  $-\log[\text{Ca}^{++}]^2[\text{HPO}_4^-]^2/[\text{Ca}_3(\text{CaPO}_4)_2][\text{aH}^+]^2 = 2.678$  (Table I).  $[\text{Ca}^{++}]$  in Series B calculated from  $\text{pK}_2, \text{CaCO}_3 = 8.007$  (3).  $-\log [\text{aH}^+] [\text{Ca}_2\text{PO}_4\text{CO}_2]/[\text{Ca}_2\text{HPO}_4\text{CO}_2] = 8.3$ .

The initial concentrations of Ca and P were 1.00 mM in Experiments 834 to 839, 0 in Experiments 931 to 935 (Table I), and 1.25 mM in all the experiments of Table II. The solid phase, in the experiments of Tables I and II, was provided by the addition of 250 mg. per liter of Preparation 147. This was obtained by the slow addition (several days) of a liter of 0.02 M  $\text{Na}_2\text{HPO}_4$  to about 3 liters of a solution containing 0.18 mole of  $\text{NaHCO}_3$ .



and 0.033 mole of  $\text{CaCl}_2$ . This material was one of several prepared by various methods in an attempt to obtain a solid phase which might represent the actual solid phase in equilibrium with bicarbonate-phosphate solutions. The attempt was unsuccessful, and no further report will be made. The various preparations were crystalline, resembling  $\text{CaHPO}_4$  in form, but had varying compositions. The particular material used in all the experiments reported in this paper contained, per 0.250 gm., 2.06 mm of Ca, 1.19 mm of P, and 0.32 mm of  $\text{CO}_2$ . It did not remain crystalline, but became amorphous and, as may be calculated from the data in Tables I and II, the final composition of the solid phase varied, the ratio of calcium to phosphorus increasing with the pH of the mixture.

It will be noted that, in Experiments  $A_4$ ,  $A_3$ , and  $A_2$ , the final concentrations of calcium and phosphorus were *greater* than the initial concentrations. The apparent greater solubility cannot, therefore, be due to delayed precipitation.

#### DISCUSSION

It was quite apparent that the solubility of calcium phosphate was increased by the presence of bicarbonate and that the solubility of calcium carbonate was increased by the presence of phosphate.

An attempt was made to interpret the results in terms of complex formation. Inasmuch as it has already been shown (3, 4) that such solutions seem to contain  $\text{CaHCO}_3^+$  and  $\text{CaHPO}_4$ , it seemed possible that the latter should dissociate  $\text{H}^+$  and that the remaining  $\text{CaPO}_4^-$  might combine with  $\text{CaHCO}_3^+$  to form a complex,  $\text{Ca}_2 \cdot \text{H} \cdot \text{PO}_4 \cdot \text{CO}_3$ . It was postulated that the mixtures were actually saturated with either  $\text{Ca}(\text{CaPO}_4)_2$  (or some substance sufficiently like this to permit the use of the product  $[\text{Ca}^{++}]^2[\text{HPO}_4^-]^2/[\text{aH}^+]^2$  as the limiting factor) or with  $\text{CaCO}_3$ . Accordingly, the  $[\text{Ca}^{++}]$  was calculated from the previously determined values of  $[\text{Ca}^{++}]^2[\text{HPO}_4^-]^2/[\text{aH}^+]^2$  (Table I), or of  $[\text{Ca}^{++}][\text{CO}_3^{--}]$  (3) in sodium chloride solutions. The lower value for  $[\text{Ca}^{++}]$  was adopted as a first approximation. With the previously established values for the instability constants of  $\text{CaHPO}_4$ ,  $\text{CaHCO}_3^+$ , and  $\text{CaCO}_3$ , first approximations to the concentrations of each of these were calculated. The difference between the concentration of total calcium and that of the sum of the ionic calcium and the three previously postulated complexes was regarded as being due to the "new complex." After the concentration of bound phosphorus and bound carbon dioxide had been subtracted from the respective totals, the calculation of the concentration of the different forms of calcium was repeated until there was no appreciable change. The values for  $-\log[\text{Ca}^{++}]^2[\text{HPO}_4^-][\text{HCO}_3^-]/[\text{Ca}_2 \cdot \text{H} \cdot \text{PO}_4 \cdot \text{CO}_3][\text{aH}^+]$  thus obtained were quite constant over a considerable range of pH, but showed a tendency to rise above pH 7.85.

This change seemed to be greater than could be accounted for by any error in analytical technique or in the choice of constants. It was recalled that  $\text{CaHCO}_3^+$  dissociates  $\text{H}^+$  and that  $-\log[\text{CaCO}_3][\text{H}^+]/[\text{CaHCO}_3^+] = 7.9$  (3). It was supposed that  $\text{Ca}_2\cdot\text{H}\cdot\text{PO}_4\cdot\text{CO}_3$  might similarly dissociate  $\text{H}^+$ . Calculations, the results of which are summarized in the last three columns of Table II, indicate that this hypothesis may be correct and that, if so, the mid-point of the dissociation is at pH 8.3. If this assumption is made, the values for  $-\log[\text{Ca}^{++}]^2[\text{HPO}_4^-][\text{HCO}_3^-]/[\text{Ca}_2\cdot\text{H}\cdot\text{PO}_4\cdot\text{CO}_3][\text{aH}^+]$  are reasonably constant over the whole range of pH, 6.90 to 9.01.

This method of calculation was applied to the data of Sendroy and Hastings obtained at 38°. For  $-\log[\text{Ca}]^2[\text{HPO}_4^-]/[\text{aH}^+]^2$ , the value 4.11 previously (5) calculated from the data of Holt, La Mer, and Chown, as recalculated by Sendroy and Hastings, was employed. The values for instability constants of  $\text{CaHPO}_4$ ,  $\text{CaHCO}_3^+$ , and  $\text{CaCO}_3$  were those obtained at room temperatures. This may have introduced a slight error. Because the values for the instability constant showed no tendency to change as the pH varied between 7.18 and 7.64 and because, even at the latter pH, the concentration of  $(\text{Ca}_2\cdot\text{PO}_4\cdot\text{CO}_3)^-$  would still be expected to be small, the formation of the latter was neglected. Although the values for the concentration of the "new complex" are obtained by difference and reflect all the errors involved in the determination of the concentrations of three different constituents and the calculation of the concentrations of three others, the values for the instability constants of this hypothetical new complex show, for ten of the eleven experiments accepted by Sendroy and Hastings,<sup>1</sup> rather less variability than do those for the product  $[\text{Ca}^{++}]^2[\text{PO}_4^-]$ .<sup>2</sup> The values for the means are  $5.75 \pm 0.165 \times 10^{-2}$  for the former, and  $3.69 \pm 0.153 \times 10^{-27}$  for the latter.

In the foregoing calculations, we have assumed that the "serum salt" solutions employed by Sendroy and Hastings did not contain magnesium. It is obvious from a comparison of their Table VIII with their Table I that this was certainly true of Experiments 7' and 7A', which appear in both the sodium chloride and "serum salt" series. Even if magnesium had originally been added, it is possible, even probable, that a considerable portion would have been precipitated, or adsorbed, onto the large amounts of solid calcium phosphate and carbonate that were added (1 gm. of each per 100 ml.). The mixtures employed by Holt, La Mer, and Chown did contain magnesium and, since, at the most, only 8.2 mm of phosphate were precipitated per liter, and no solid phase was added as such, probably very little magnesium was precipitated with the calcium phosphate. Therefore,

<sup>1</sup> Experiment 15 was omitted because an accurate value for the instability constant cannot be calculated because the concentration of phosphorus is so low that five-sixths of it is bound as complex, leaving only  $10^{-4} \text{ M}$  present as free phosphate.

there must have been present, in solution,  $\text{MgHCO}_3^+$ ,  $\text{MgCO}_3$ , and  $\text{MgHPO}_4$ . There was also the possibility of the formation of a complex similar to that postulated for calcium and of mixed complexes as well.

The former of these possibilities was investigated by the determination of the solubility of magnesium phosphate in the presence and absence of bicarbonate. The apparent solubility product of  $\text{MgHPO}_4$  had previously been determined by Tabor and Hastings (6) and the negative logarithm found to be  $4.46 \pm 0.03$ ,<sup>2</sup> at  $\mu \approx 0.16$  and at  $38^\circ$ , over a rather narrow range of pH and of phosphate concentration. As may be seen from Tables III and IV, our values over a far greater range of pH, phosphate, and magnesium concentrations are similar to that of Tabor and Hastings, and are almost identical in the presence and in the absence of bicarbonate,  $4.225 \pm 0.067$  and  $4.12 \pm 0.03$ , respectively. The failure to find any such difference as was observed with calcium phosphate may appear rather surprising, but it must be remembered that magnesium carbonate and phosphate differ in other respects from the corresponding calcium compounds. Magnesium carbonate dissociates in water to form basic salts and even the hydroxide, while calcium carbonate does not. On the other hand, the apparent solubility product for  $\text{MgHPO}_4$  is constant even up to pH 7.8 (Table III) or pH 8.0 (Table IV), whereas with calcium the corresponding product fails at least 2 pH units below this and the precipitate obtained contains calcium and phosphorus in the atomic ratio of 1.5, or more, to 1.

In calculations from the data of Holt, La Mer, and Chown, allowance was made for the formation of  $\text{MgHCO}_3^+$ ,  $\text{MgCO}_3$ , and  $\text{MgHPO}_4$ , but not for the existence of mixed complexes containing both magnesium and calcium. The apparent instability constants thus found varied between  $2.1 \times 10^{-2}$  and  $4.7 \times 10^{-2}$ , with an average of  $2.9 \times 10^{-2}$ . These are somewhat smaller than in the case of the experiments of Sendroy and Hastings. This difference may be the result of a systematic error due to differences in technique in the two laboratories or may, indeed, indicate the real existence of mixed complexes.

It may occur to the reader that the hypothesis developed in this paper requires the assumption of the existence of five new substances and of five instability constants. In a sense that is true. However, the existence of three of these complexes and the validity of the corresponding constants have been established in previous publications (3, 4), and the existence of  $\text{MgHPO}_4$  and the value of its instability constant have been verified by Tabor and Hastings (6). Therefore, it is now necessary to assume only the existence of a new pair of complexes, related to each other as acid and anion.

<sup>2</sup> The calculation of the standard deviation is ours

TABLE III  
Solubility of Magnesium Phosphate in Absence of Bicarbonate

Experiment No.	Equilibrated	pH	Mg	P	$pK'_{sp}MgHPO_4$	$\mu \times 10^3$
	days		moles $\times 10^3$	moles $\times 10^3$		
1	6	7.77	9.88	8.42	4.26	151
2	6	7.81	10.65	9.10	4.20	148
3	6	7.40	20.7	9.24	3.97	163
4	10	7.43	13.1	11.1	4.08	128
5	11	7.11	14.5	12.8	4.04	124
6	11	6.90	14.8	12.7	4.09	129
7	11	6.91	18.5	12.25	4.01	136
8	11	6.82	19.4	11.55	4.05	138
41	6	7.10	4.64	35.85	4.19	149
42	6	7.10	4.26	35.45	4.23	149
43	6	7.03	3.84	36.5	4.28	155
44	6	6.90	4.26	33.8	4.30	149
109B	15	6.33	51.0	12.25	3.90	149
109C	15	6.33	56.3	6.13	4.14	165
Average, calculated from average for ion product. .					$4.12 \pm 0.03$	

TABLE IV  
Solubility of Magnesium Phosphate in Presence of Bicarbonate

Experiment No.	Equilibrated	pH	Mg	P	CO <sub>2</sub>	pK' <sub>sp</sub> MgHPO <sub>4</sub>	$\mu \times 10^3$
	days		moles $\times 10^3$	moles $\times 10^3$	moles $\times 10^3$		
1	28	6.78	57.0	2.43	20.2	4.41	157
2	28	6.30	52.9	3.60	33.2	4.42	156
4	32	6.40	40.8	2.85	7.31	4.54	121
5	32	6.88	42.0	2.00	17.25	4.47	125
6	35	6.80	41.3	2.18	14.0	4.47	122
11	11	8.00	9.38	7.94	51.6	4.39	146
12	11	7.80	14.0	7.90	38.8	4.21	147
13	20	7.69	10.1	9.10	30.3	4.28	135
14	20	7.40	12.3	10.8	10.4	4.16	125
21	19	7.30	8.86	8.34	52.0	4.44	141
22	14	7.22	10.1	9.17	41.3	4.45	136
23	14	7.12	10.75	9.86	31.7	4.41	130
24	14	6.80	14.6	11.9	14.4	4.18	125
51	14	7.32	10.1	40.0	50.7	3.77	166
52	14	7.20	7.93	38.5	51.5	3.99	157
53	14	7.22	6.13	35.2	50.5	4.11	155
54	14	7.20	5.92	33.7	63.5	4.05	157
Average, calculated from average for ion product .						4.225 $\pm$ 0.067	

## SUMMARY

The solubility of calcium carbonate at  $\mu \approx 0.155$  is increased by the presence of phosphate, and that of calcium phosphate is increased by the presence of bicarbonate. Data are presented that indicate the existence of the complexes  $(\text{Ca}_2 \cdot \text{H} \cdot \text{PO}_4 \cdot \text{CO}_3)$  and  $(\text{Ca}_2 \cdot \text{PO}_4 \cdot \text{CO}_3)^-$ , with  $-\log [a\text{H}^+][(\text{Ca}_2 \cdot \text{PO}_4 \cdot \text{CO}_3)^-]/[\text{Ca}_2 \cdot \text{H} \cdot \text{PO}_4 \cdot \text{CO}_3] = 8.3$ .

Over a considerable range of calcium, phosphorus, and hydrogen ion concentration, the value for  $-\log [\text{Ca}^{++}]^2 [\text{HPO}_4^-] [\text{HCO}_3^-] / [\text{Ca}_2 \cdot \text{H} \cdot \text{PO}_4 \cdot \text{CO}_3] [a\text{H}^+] = 1.33$ . The hypothesis is shown to be consistent with data previously reported by others.

The apparent solubility product for  $\text{MgHPO}_4$  has been determined over a wide range of concentrations of magnesium, phosphate, and hydrogen ions and found to be the same in the presence, as in the absence, of bicarbonate.

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# THE MICROBIOLOGICAL DETERMINATION OF AMINO ACIDS IN ANIMAL PROTEINS

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The amino acid requirements of the lactic acid bacteria have been studied by numerous investigators and adaptation to the assay of amino acids has been proposed. Shankman *et al.* (1) reported that arginine, glutamic acid, leucine, phenylalanine, tryptophane, tyrosine, and valine in synthetic mixtures could be determined by this procedure. Arginine, valine, leucine, isoleucine, phenylalanine, and glutamic acid have been determined in casein and other proteins with the organisms *Lactobacillus arabinosus* or *Lactobacillus casei* by Dunn *et al.* (2), Lyman *et al.* (3), Kuiken *et al.* (4), McMahan and Snell (5), Hegsted (6), Schweigert *et al.* (7), and Lewis and Olcott (8). Lysine has been determined in proteins with the organism *Leuconostoc mesenteroides* by Dunn *et al.* (9). These workers also suggested that this organism could be used to determine other amino acids as well (10).

We have investigated the microbiological amino acid determination of arginine, histidine, lysine, leucine, isoleucine, valine, tyrosine, phenylalanine, threonine, and glutamic acid in eight representative animal proteins. A method for the preparation of the samples for assay is described and evidence of the reproducibility and reliability of the microbiological amino acid assays of animal protein is presented.

## EXPERIMENTAL

*Standard Solutions*—Owing to the variations in purity of natural amino acids, synthetic racemic mixtures were used wherever possible. Hegsted (11) has reported that *Lactobacillus arabinosus* utilizes only the *l* form of isoleucine and valine, while the utilization of *d*-leucine is so slight as to be unimportant. We also found that *dl*-lysine had activity equal only to the *l*-lysine content for *Leuconostoc mesenteroides*. It was therefore assumed that only the *l* form of the racemic mixtures used as standards was active and the results are reported in terms of *l*-amino acid composition. All standard solutions were preserved under toluene in the refrigerator. The following amino acids were used in preparing standard solutions: *dl*-isoleucine (Merck), *dl*-leucine (Merck), *dl*-threonine (Merck), *l*(+)-glutamic acid, *dl*-valine (Merck), *dl*-phenylalanine (Merck), *l*(+)-arginine hydrochloride (Pfanstiehl), *l*(-)-tyrosine (Pfanstiehl), *l*(-)-histidine hydro-

chloride + H<sub>2</sub>O (analytical purity grade, Amino Acid Manufactures), and *dl*-lysine hydrochloride (Merck).

### Procedure

*Glutamic Acid, Leucine, Isolucine, Valine, and Threonine*—These amino acids were determined with *Lactobacillus arabinosus* 17-5, the basal medium described in Table I being used. This medium is modified according to the procedure of Shankman (12), and the usual microbiological technique of

TABLE I

*Medium\* for One Hundred Tube Assay for Leucine, Isolucine, Valine, Threonine, or Glutamic Acid*

The amino acid being assayed is omitted from the basal medium.

	cc.		mg.
Salts A.....	5	<i>l</i> (-)-Tyrosine.....	40
" B.....	5	<i>dl</i> -Phenylalanine.....	100
	cm.	<i>l</i> (+)-Lysine HCl.....	200
Glucose.....	20	<i>dl</i> -Alanine.....	200
Sodium acetate.....	20	<i>l</i> (+)-Arginine HCl.....	50
Ammonium sulfate†.....	6	<i>l</i> (-)-Histidine HCl·H <sub>2</sub> O.....	50
	γ	Asparagine.....	400
Biotin.....	0.4	<i>l</i> (-)-Proline.....	100
	mc.	<i>dl</i> -Serine.....	50
Adenine H <sub>2</sub> SO <sub>4</sub> .....	10	<i>dl</i> -Norleucine.....	100
Guanine HCl.....	10	<i>l</i> (-)-Hydroxyproline.....	100
Uracil.....	10	Glycine.....	100
Thiamine HCl.....	0.20	<i>dl</i> -Isoleucine.....	200
Niacin.....	0.20	<i>dl</i> -Leucine.....	200
Ca pantothenate.....	0.20	<i>dl</i> -Threonine.....	200
Pyridoxine HCl.....	0.20	<i>l</i> (+)-Glutamic acid.....	400
<i>p</i> -Aminobenzoic acid.....	0.10	<i>dl</i> -Valine.....	200
Riboflavin.....	0.20	<i>l</i> (-)-Cystine.....	100
<i>l</i> (-)-Tryptophane... ..	40	<i>dl</i> -Methionine.....	100

\* Modified according to the procedure of Shankman (12).

† Included only for the glutamic acid assay.

inoculation, incubation, and titration with 0.1 N sodium hydroxide was followed (2, 12, 13). It was found that the autoclaving time was quite important inasmuch as excessive autoclaving led to low maximum growth and unsatisfactory assays. This was particularly true for the threonine assays in which excessive autoclaving gave high blanks and made it impossible to obtain an assay. This was probably due to the formation of pyridoxal or pyridoxamine, as was reported by Stokes and Gunness (14), enabling the organism to synthesize threonine. Titration ranges from a blank of 1.1 to 1.4 cc. to a maximum of 13 to 14 cc. were usual for 0 to 200

$\gamma$  of *dl*-leucine, *dl*-isoleucine, *dl*-valine, and 0 to 300  $\gamma$  of *l*-glutamic acid, while 2.4 to 2.6 up to 13 to 14 cc. was the usual range for 0 to 200  $\gamma$  of *dl*-threonine.

*Arginine, Phenylalanine, and Tyrosine*—These amino acids were determined with *Lactobacillus casei*, with the medium described by McMahan and Snell (5). Arginine, phenylalanine, and tyrosine were omitted from the stock solutions and the two not being assayed were then added in solid form to the complete basal medium. 10 cc. volumes were used and results were obtained by titration. Titration ranges of 1.0 to 9.0 cc. of 0.1 *N* sodium hydroxide were usually obtained for 0 to 200  $\gamma$  of *l*-arginine and *dl*-phenylalanine and 0 to 100  $\gamma$  of *l*-tyrosine.

*Histidine and Lysine*—These amino acids were assayed with *Leuconostoc mesenteroides* P-60 with the improved Medium D described by Dunn *et al.* (9, 10). Histidine and lysine were omitted from the stock solutions and the one not being assayed was added in solid form to the complete basal medium. Results were obtained by titration. Titration ranges of 1.0 to 9.0 cc. were usually obtained for 0 to 100  $\gamma$  of *l*-histidine and 0 to 500  $\gamma$  of *dl*-lysine.

*Proteins Assayed*—Nitrogen values are reported on the ash-, water-, and fat-free basis.

*Casein*—Labco casein, nitrogen content 15.6 per cent.

*Fibrin*—Wilson's fibrin, a commercial product prepared from fresh beef blood by stirring. The crude clot was washed free of hemoglobin and cells with water, extracted with fat solvents, and vacuum-dried. Nitrogen content 16.6 per cent.

*Lactalbumin*—Borden's lactalbumin 15-42 which probably also contained lactoglobulin. Nitrogen content 14.3 per cent.

*Gelatin*—Wilson's pure food gelatin, a pork skin type of gelatin. Nitrogen content 17.6 per cent.

*Pancreas*—Wilson's pancreas, dried defatted pork pancreas. Nitrogen content 15.4 per cent.

*Beef Muscle*—The gluteus maximus muscle of a dairy cow was trimmed of its outside fat, hashed, and desiccated with 10 volumes of acetone. The liquid was separated by filtration and the meat washed twice with 2 liter portions of acetone. The residue was then dried *in vacuo*. This residue was then exhaustively extracted with ether by Soxhlet extraction and again dried. Nitrogen content 16.4 per cent.

*Bovine Albumin*—Crystalline bovine serum albumin, Armour.<sup>1</sup> Nitrogen content 16.02 per cent.

<sup>1</sup> Kindly supplied by Dr. F. C. Koch of the Armour Research Laboratories, Chicago, Illinois.



*Beef Serum*—After defibrination of fresh beef blood by stirring, the liquid portion was centrifuged to remove cells, and the supernatant serum was spray-dried. Nitrogen content 14.9 per cent.

TABLE II  
*Effect of Hydrolysis Time with 8 N HCl on Amino Acid Assay*

Amino acid	Fibrin		Pancreas		Casein		
	8 hrs.	24 hrs.	4 hrs.	16 hrs.	4 hrs.	8 hrs.	24 hrs.
	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Arginine.....	7.1	7.2	6.4	6.7	3.6	3.5	3.7
Glutamic acid.....	15.5	14.4	6.2	6.1	20.5	21.4	22.2
Histidine.....	2.2	2.2	1.4	1.4	2.6	2.4	2.7
Isoleucine.....	5.9	5.9	5.8	5.8	5.6	5.6	6.0
Leucine.....	6.8	6.9	7.0	7.0	9.8	9.5	9.2
Lysine.....	8.2	8.6	6.4	6.5	7.2	7.2	7.1
Phenylalanine.....	4.6	4.2	3.8	3.8	5.5	5.2	5.4
Threonine.....	6.1	6.7	5.8	5.2	4.2	4.3	4.2
Tyrosine.....	6.3	5.8	4.1	4.1	6.7	6.5	6.4
Valine.....	5.1	5.8	5.2	5.3	7.0	7.4	7.5

TABLE III  
*Comparison of Results of Three Separate Assays of Lactalbumin and Beef Muscle over 3 Month Period*

Amino acid	Lactalbumin			Mean and maximum deviation from mean	Maximum per cent deviation from mean	Beef muscle			Mean and maximum deviation from mean	Maximum per cent deviation from mean
	As-say 1	As-say 2	As-say 3			As-say 1	As-say 2	As-say 3		
	per cent	per cent	per cent			per cent	per cent	per cent		
Arginine.....	3.1	3.2	3.3	3.2 ± 0.1	3.1	6.0	5.6	6.3	6.0 ± 0.4	6.7
Glutamic acid..	17.2	16.8	16.2	16.8 ± 0.6	3.6	14.8	15.0	15.8	15.2 ± 0.6	3.9
Histidine.....	1.8	1.9	1.7	1.8 ± 0.1	5.6	3.7	3.7	3.3	3.5 ± 0.2	5.7
Isoleucine.....	6.3	5.8	5.9	6.0 ± 0.3	5.0	5.5	5.1	5.3	5.3 ± 0.2	3.8
Leucine.....	10.8	11.1	10.6	10.8 ± 0.3	2.5	7.9	7.6	7.6	7.7 ± 0.2	2.6
Lysine.....	8.5	8.3	8.5	8.4 ± 0.1	1.9	7.3	8.3	8.1	7.9 ± 0.6	7.6
Phenylalanine..	3.9	3.5	3.7	3.7 ± 0.2	5.4	3.9	3.8	4.0	3.9 ± 0.1	2.6
Threonine.....	5.4	5.1	6.1	5.6 ± 0.5	8.9	5.8	5.1	5.2	5.4 ± 0.4	7.4
Tyrosine.....	4.9	4.6	4.2	4.6 ± 0.4	8.7	4.0	4.3	4.1	4.1 ± 0.2	4.8
Valine.....	6.2	5.6	6.2	5.9 ± 0.3	5.1	5.2	5.2	5.2	5.2 ± 0.0	0.0

*Preparation of Samples for Assay*—2 gm. of sample were refluxed with 80 cc. of 8 N hydrochloric acid for 8 hours in all-glass equipment. The hydrolysate was then diluted to roughly 500 cc. with distilled water and neutralized with 8 N sodium hydroxide to pH 6.8, bromothymol blue being

used. The volume was accurately adjusted to 1000 cc. with distilled water and the solution was filtered. The filtrate was then diluted, if necessary, so that the concentration of the amino acid being assayed was approximately 20  $\gamma$  per cc.

Evidence that 8 hours hydrolysis time with 8 N hydrochloric acid is adequate for fibrin, pancreas, and casein is presented in Table II. No destruction of the amino acids occurred when hydrolysis was conducted for 24 hours, while even 4 hours of hydrolysis gave approximately the same values. Similar results were obtained with the other proteins reported.

TABLE IV

*Recovery of Amino Acids Added to 2 Gm. of Lactalbumin or Beef Muscle Previous to Hydrolysis*

Amino acid	Lactalbumin				Beef muscle			
	Amount in sample by assay*	l isomer added	Amino acid recovered	Recovery	Amount in sample by assay*	l isomer added	Amino acid recovered	Recovery
	mg.	mg.	mg.	per cent	mg.	mg.	mg.	per cent
Arginine.....	57	42†	42	100	106	42†	42	100
Glutamic acid.....	284	300	310	103	293	300	273	91
Histidine.....	30	34‡	34	100	69	34‡	30	88
Isoleucine.....	100	100§	108	108	103	100§	93	93
Leucine.....	186	208§	200	96	147	200§	199	100
Lysine.....	150	120	118	98	154	120	121	100
Phenylalanine.....	64	75§	74	99	76	75§	75	100
Threonine.....	106	100§	114	114	95	100§	95	95
Tyrosine.....	85	40	38	95	81	40	41	103
Valine.....	107	100§	108	108	115	100§	100	100

\* These values are reported as found without correction for the ash, water, or fat content of the protein sample.

† Added as l(+)-arginine hydrochloride (50 mg.).

‡ Added as l(-)-histidine hydrochloride + H<sub>2</sub>O (46 mg.).

§ Added as the dl mixture in twice the amount indicated.

|| Added as dl-lysine hydrochloride (300 mg.).

**Reproducibility**—To test the reproducibility of values from one test to another, three separate assays for lactalbumin, casein, and beef muscle were conducted. The maximum percentage deviation from the mean was found to be less than 10 per cent in the case of all of the amino acids reported. Table III shows typical results obtained in the tests made with lactalbumin and beef muscle.

**Recovery Experiments**—Table IV shows the results of recovery experiments with lactalbumin and beef muscle. The recoveries range from 88 to 114 per cent with a mean of 100 per cent for all the recovery experi-

TABLE V

Arginine, Histidine, and Lysine Content of Proteins

The figures in parentheses indicate the nitrogen content of the protein sample on which the assay value is based.

Protein	Arginine			Histidine			Lysine		
	Microbiological value*		Reference No.	Chemical value		Reference No.	Microbiological value*		Reference No.
	per cent	per cent		per cent	per cent		per cent	per cent	
Casein	3.6	3.7 (15.5)	15	2.6	2.4 (15.5)	16	7.6	7.8 (13.8)	17
	3.7 (?)	3.8 (14.5)	18		1.7 (15.2)	19	7.7 (14.2)	7.3 (15.5)	16
Beef fibrin	7.2	8.1 (17.0)	21	2.2	2.5 (15.8)	20	8.5	9.1 (15.2)	17
		8.2 (16.8)	15		2.4 (17.0)	21			
Lactalbumin	3.2	3.5 (14.2)	18	1.8	2.5 (17.0)	16	8.4	6.2 (14.0)	22
		3.6 (14.9)	22		2.06 (16.8)	20		8.3 (13.8)	16
Gelatin	8.0	3.8 (15.5)	16		2.2 (14.2)	19	4.1	8.1 (14.2)	19
	9.3 (?)	7.0 (16.0)	16	0.79	1.4 (14.9)	22		4.3 (16.0)	16
Pork pancreas	6.6	9.3 (16.0)	23		0.51 (18.3)			5.3 (?)	17
Beef muscle	6.0	6.1† (16.0)	16	1.4	2.7† (16.0)	16	6.5	5.1† (16.0)	16
		7.1 (16.0)	16	3.5	2.2 (16.0)	16	7.9	8.0 (16.0)	16
Bovine albumin	6.1	6.9 (16.0)	24		2.2 (16.0)	24	12.3	8.1 (16.0)	24
		6.1 (16.07)	21	4.1	3.83 (16.07)	21		6.5 (16.07)	21
Beef serum	5.3	4.8 (14.2)	26	1.8	1.2 (14.2)	26	7.3	12.42 (16.07)	25
								6.9 (14.2)	26

\* The first value for each protein is that of the authors reported on the ash-, water-, fat-free basis.

† Beef pancreas value.

ments, indicating that recoveries are quantitative and that the hydrolysis procedure as well as the assay procedure itself is satisfactory for all of these assays.

*Validity of Procedures*—Tables V to VIII show the results of the microbiological assay of the proteins described above. These values are com-

TABLE VI  
*Threonine and Glutamic Acid Content of Proteins*

The figures in parentheses indicate the nitrogen content of the protein samples on which the assay value is based

Protein	Threonine			Glutamic acid			
	Microbiological value*	Chemical value		Microbiological value*		Chemical value	
			Ref- erence No		Ref- erence No		Ref- erence No
	per cent	per cent		per cent		per cent	
Casein	4 1	3 5 (14 0)	27	21 4		22 8 (16 0)	16
		3 5 (14 7)	16	22 5 (15 6)	2	22 0 (15 7)	28
				19 7 (14 0)	8	22 0 (14 0)	29
Beef fibrin	6 4			21 5 (15 2)	3		
		7 9 (16 0)	16	15 0		13 1 (15 2)	30
		6 8 (17 0)	21	12 4 (16 0)	8	16 0 (16 0)	29
Lactalbumin	5 6	4 5 (13 8)	16	16 8		13 7 (14 9)	22
		4 3 (14 9)	22			13 4 (16 0)	16
		5 2 (15 4)	31				
Gelatin	1 9	1 5 (16 0)	16	11 5		12 0 (18 0)	29
		1 4 (16 0)	27	10 2 (18 0)	8		
Pork pancreas	5 6	4 1† (16 0)	16	6 2			
Beef muscle	5 4	5 3 (16 0)	16	15 2		15 4 (16 0)	16
		4 6 (16 0)	24				
Bovine albumin	6 3	6 46 (16 07)	21	18 0		16 9 (16 07)	21
						16 95 (16 07)	25
Beef serum	5 9	6 3‡ (16 0)	16	13 2			

\* The first value for each protein is that of the authors reported on the ash-, water-, fat-free basis

† Beef pancreas value

‡ Human serum protein value

pared with chemical and microbiological values reported in the literature.

*Arginine, Histidine, and Lysine (Table V)*—The microbiological values agree satisfactorily with chemical values for arginine, histidine, and lysine in most cases. Unsatisfactory agreement appears in the histidine assay of pancreas and beef muscle and the lysine assay for pancreas. Our value for the lysine content of bovine albumin agrees quite well with that of

Shemin (25) obtained by the isotope dilution method. Brand's value (21) obtained by the Van Slyke manometric method is considerably lower.\*

*Threonine and Glutamic Acid (Table VI)*—Snell (43) has shown that pyridoxamine and pyridoxal are present in natural products, particularly in liver. Since *Lactobacillus arabinosus* can synthesize threonine in the

TABLE VII

*Tyrosine and Phenylalanine Content of Proteins*

The figures in parentheses indicate the nitrogen content of the protein samples on which the assay value is based.

Protein	Tyrosine			Phenylalanine		
	Microbiological value*	Chemical value		Microbiological value*	Chemical value	
			Reference No.			Reference No.
	<i>per cent</i>	<i>per cent</i>		<i>per cent</i>	<i>per cent</i>	
Casein	6.5	6.5 (15.1)	32	5.3	5.0 (15.4)	33
		6.4 (16.0)	16		4.0 (?)	34
		6.0 (14.5)	18			
Beef fibrin	6.0	5.0 (16.0)	16	4.4	6.0 (16.0)	16
		5.7 (17.0)	21			
Lactalbumin	4.6	4.7 (14.2)	18	3.7	4.8 (13.8)	16
		3.5 (14.9)	22		4.5 (14.9)	22
					3.6 (?)	34
Gelatin	0.44	0.3 (16.0)	16	2.2	1.9 (16.0)	16
		0.4 (16.0)	35		2.5 (?)	36
					1.6 (?)	34
Pork pancreas	4.2	3.0† (16.0)	16	3.8	4.4‡ (16.0)	16
Beef muscle	4.1	3.4 (16.0)	16	3.9	4.9 (16.0)	16
		4.3 (16.0)	24		4.9 (16.0)	24
Bovine albumin	5.2	5.49 (16.07)	21	6.4		
		5.53 (16.07)	25			
Beef serum	4.9	4.7 (16.0)	16	4.7	5.4‡ (16.0)	16

\* The first value for each protein is that of the authors reported on the ash-, water-, fat-free basis.

† Beef pancreas value.

‡ Human serum protein value.

presence of either of these substances, the possibility exists that threonine values obtained microbiologically may be high. However, the agreement between our values and those obtained chemically would indicate that

\* Since this paper was submitted for publication Brand and coworkers have reported a value of 12.4 per cent lysine in bovine serum albumin obtained by microbiological assay with *Leuconostoc mesenteroides* P-60 (*J. Am. Chem. Soc.*, **67**, 1524 (1945)). This is in excellent agreement with our value of 12.3 per cent.

TABLE VIII  
*Leucine, Isoleucine, and Valine Content of Proteins*

The figures in parentheses indicate the nitrogen content of the protein samples on which the assay value is based.

Protein	Leucine				Isoleucine				Valine			
	Microbiological value*		Chemical value		Microbiological value*		Chemical value		Microbiological value*		Chemical value	
	per cent	Reference No.	per cent	Reference No.	per cent	Reference No.	per cent	Reference No.	per cent	Reference No.	per cent	Reference No.
Casein	9.5		12.1 (10.0)	10	6.0		5.2 (15.3)	22	7.1		7.0 (15.0)	37
	9.8 (?)	38			6.1 (?)	4			0.3 (?)	4	7.0 (10.0)	10
	9.3 (?)	4							0.7 (?)	5		
Beef fibrin	6.9		14.3 (10.0)	10	5.9		5.0 (10.0)	10	5.5		3.9 (10.0)	10
	7.5 (17.0)	21										
	10.8		17.4 (14.9)	22	6.0		4.2 (14.9)	22	5.9		4.0 (14.9)	22
Lactalbumin	3.2		3.5 (18.3)	30	1.4		1.1 (16.0)	10	2.5		2.3 (10.0)	40
	3.3 (?)	1	3.7 (10.0)	10	1.7 (?)	4			2.5 (?)	4		
	3.6 (18.3)	38							2.7 (?)	5		
Pork pancreas	7.0		0.0† (16.0)	11	5.9		3.4† (10.0)	41	5.3		4.2† (10.0)	41
Beef muscolo	7.7		12.1 (16.0)	16	5.3		3.4 (10.0)	10	5.2		3.4 (10.0)	10
Bovine albumin	7.9 (16.0)	42			5.9 (10.0)	40			5.2 (10.0)	42		
	11.3		13.7 (16.07)	21	3.3				5.4			
Beef serum	9.4				3.2				7.0			

\* The first value for each protein is that of the authors reported on the ash-, water-, fat-free basis.

† Beef pancreas value.

these substances are not present in our samples in sufficient quantity to produce this stimulatory effect.

The glutamic acid results show good agreement between microbiological and chemical assays as previously reported by Lewis and Oleott (8), Dunn *et al.* (2), and Lyman *et al.* (3). There is also good agreement between the various laboratories for the microbiological assay of similar proteins.

*Tyrosine and Phenylalanine (Table VII)*—The microbiological and chemical values for tyrosine agree satisfactorily in all samples except in the case of pancreas. The agreement between microbiological and chemical values for phenylalanine is not very satisfactory. However, the poor agreement between values obtained chemically indicates that some of the chemical values lack accuracy. The microbiological value of 3.70 per cent phenylalanine in casein reported by Hegsted (6) is not included in Table VII inasmuch as the values he reported for leucine and valine in casein were also lower than those obtained by other workers, indicating his media to have been deficient.

*Leucine, Isoleucine, and Valine (Table VIII)*—There is good agreement between our values for leucine, isoleucine, and valine and those reported by other workers using *Lactobacillus* microbiological procedures. Ryan and Brand (21, 38) reported the leucine content of casein, fibrin, and gelatin using the *Neurospora* mutant spore procedure. Our values for these proteins agree quite well with theirs. The gelatin sample of Ryan and Brand is apparently the same preparation used by Stein (39) who reported 3.5 per cent by the solubility product procedure. Good agreement between all three methods is therefore indicated.

The chemical oxidation procedure of Bloek (16) appears to give considerably higher values for leucine and lower values for isoleucine and valine than the microbiological procedures. Gordon *et al.*, however, report 2.3 per cent valine in gelatin (40) determined chromatographically, which agrees quite well with our figure of 2.5 per cent.

#### DISCUSSION

The application of the microbiological assay to the determination of amino acids offers a rapid, convenient, and accurate procedure for the study of protein composition. Our microbiological values agree quite well with values obtained by chemical procedures, and it is possible for one analyst to determine all of the amino acids described in this paper in four to five samples in approximately 7 working days. This is a considerable improvement over the older chemical procedures in time saved and in equipment required as well as in accuracy and reproducibility.

McMahan and Snell (5) indicated that while all of the amino acids used in their basal medium are not essential for growth of the organism heavier

growth is obtained and antagonistic effects occur less frequently when complete amino acid mixtures are used. We wish to confirm these observations and indicate that, with the media reported in this paper, we have found no indications of antagonistic effects despite the varied samples studied. In addition, after 72 hours incubation and with the titration procedure, there was no indication of drift in values at different levels of the sample which has been reported for certain of the microbiological vitamin assays. Generally, the agreement between levels of the sample when calculated to micrograms of amino acids per cc. is well within 10 per cent and usually within 5 per cent.

#### SUMMARY

A simple method for preparing samples for the microbiological assay of certain amino acids is described. The hydrolysate obtained may be assayed for arginine, histidine, lysine, leucine, isoleucine, valine, threonine, tyrosine, glutamic acid, and phenylalanine by methods described. The amounts of these ten amino acids in casein, beef fibrin, lactalbumin, gelatin, pork pancreas, beef muscle, crystalline bovine albumin, and beef serum are reported. Evidence of reproducibility and of the validity of these values is presented.

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# MICROBIOLOGICAL DETERMINATION OF FREE LEUCINE, ISOLEUCINE, VALINE, AND THREONINE IN DOG PLASMA

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Considerable work on the application of microbiological procedures to the determination of amino acids in proteins has been reported in the recent literature. Methods for the determination of leucine, isoleucine, and valine have been described by Shankman *et al.* (1), Hegsted (2), Schweigert *et al.* (3), and Hier *et al.* (4). The latter authors also described a method for the determination of threonine in animal proteins. In work preliminary to studies on the metabolism of proteins we have successfully applied these methods to the determination of free leucine, isoleucine, valine, and threonine in dog plasma. Our procedure follows along the lines suggested by Dunn *et al.* (5) for the determination of apparent free tryptophane in human blood.

## EXPERIMENTAL

*Preparation of Tungstic Acid Filtrate*—40 to 50 cc. of blood were removed from the femoral artery of mongrel dogs previously fasted for 24 hours. Clotting was prevented by the use of heparin. Centrifugation and removal of the plasma were carried out within 1 hour of collection. For the determination of leucine, isoleucine, valine, and threonine 16.0 cc. of plasma were measured into a flint glass 125 cc. bottle, followed by 22.4 cc. of distilled water. The bottle was then stoppered and shaken, and 5.6 cc. of exactly 0.60  $N$   $H_2SO_4$  were added slowly with mixing, followed by 4.0 cc. of exactly 10.0 per cent sodium tungstate. The stoppered bottle was then shaken for 2 to 3 minutes and the fluid was filtered through Whatman No. 40 paper. A water-white, clear filtrate was obtained with all plasma samples when this procedure was used. The pH was then adjusted with 0.7  $N$  NaOH to 6.8, bromothymol blue being used as an outside indicator. 2 to 4 drops of sodium hydroxide were usually adequate, thus having a negligible effect on the volume. Each cc. of tungstic acid filtrate, therefore, represented 0.33 cc. of original plasma.

*Assay Procedure*—The basal media were prepared as described in a previous paper by Hier *et al.* (4). Small tubes (16  $\times$  150 mm.) were used and only 1.0 cc. of basal medium was added to each tube. After the addition of standard and unknown solutions the final volume was adjusted to

2.0 cc.; the tubes were plugged and sterilized for 10 minutes. All other details were carried out as previously described (4) except that the tubes were titrated with 0.02 N NaOH. The standard curves for leucine, isoleucine, and valine with levels from 0 to 30  $\gamma$  per tube ranged from about

TABLE I

*Recovery of Leucine, Isoleucine, Valine, and Threonine Added to Plasma Previous to Precipitation of Proteins*

Plasma sample No.	Found per cc. plasma	Added per cc. plasma*	Recovered	Recovery	Found per cc. plasma	Added per cc. plasma*	Recovered	Recovery
Leucine					Isoleucine			
	$\gamma$	$\gamma$	$\gamma$	per cent	$\gamma$	$\gamma$	$\gamma$	per cent
1	13.7	10.5	11.0	105	9.0	10.0	12.5	125
2	15.6	10.0	9.0	90	10.0	10.4	10.5	100
3	13.5	16.0	16.0	100	9.5	20.0	16.0	80
4	14.4	20.0	17.0	85	9.2	20.0	21.0	105
5	16.4	20.0	19.5	98	11.0	20.0	18.0	90
6	20.0	20.0	18.8	94	15.6	20.0	20.5	103
7	20.9	20.0	21.5	108	18.0	25.0	22.5	90
8	22.5	25.0	27.0	108	12.6	20.0	21.5	108
Average recovery and standard deviation.....				98.5 $\pm$ 8.4	100 $\pm$ 13.7			
Valine					Threonine			
1	21.2	10.0	12.5	125	30.0	10.4	12.0	115
2	17.7	10.4	9.7	93	40.5	20.0	16.1	81
3	19.5	6.2	6.1	98	32.1	20.0	19.0	95
4	19.0	20.0	23.0	115	18.0	20.0	18.0	95
5	20.4	20.0	20.0	100	33.0	20.0	17.0	85
6	17.0	20.0	19.0	95	31.5	25.0	22.5	90
7	16.0	20.0	20.0	100				
8	18.0	20.0	19.0	95				
9	24.0	20.0	17.5	89				
10	24.6	25.0	26.0	104				
Average recovery and standard deviation.....				102 $\pm$ 10.8	93.5 $\pm$ 11.9			

\* Added as the *dl* form in twice the amount indicated.

1.0 to 13.0 cc. The threonine curve with the same levels ranged from about 2.0 to 13.0 cc.

The standard solutions were prepared with Merck's *dl*-leucine, *dl*-isoleucine, *dl*-valine, and *dl*-threonine. All values are reported in terms of the *l*-amino acid content of the racemic standards (50 per cent).

Agreement between values at different levels was well within 10 per cent of the mean in all cases. There was no evidence of drift due to inhibition

TABLE II

*Reproducibility of Assays and Effect of Storage of Plasma at -17°*

Dog No.	Days storage	Amino acid found	Dog No.	Days storage	Amino acid found
Leucine			Isoleucine		
		<i>γ per cc.</i>			<i>γ per cc.</i>
2	0	13.7	2	0	9.0
2	0	15.6	2	15	10.0
		$14.7 \pm 1.4^*$			$9.5 \pm 0.7^*$
3	0	13.5	3	0	9.5
3	15	14.4	3	14	9.2
3	22	16.4	3	21	9.9
3	30	15.0			$9.5 \pm 0.4^*$
		$14.7 \pm 1.3^*$			
4	0	20.0	4	0	11.0
4	8	19.5	4	8	15.0
4	8	18.0	4	8	15.0
		$19.2 \pm 1.1^*$			$13.7 \pm 2.3^*$
5	0	22.7	5	0	14.7
5	0	23.7	5	0	14.3
5	7	20.9	5	7	15.6
		$22.4 \pm 1.0^*$			$14.9 \pm 0.7^*$
Threonine			Valine		
2	0	30.0	1	1	21.7
2	7	30.0	1	1	21.2
		$30.0 \pm 0.0^*$			$21.5 \pm 0.4^*$
3	0	40.5	3	1	19.5
3	7	32.1	3	7	19.0
3	15	32.0	3	14	20.4
		$35.0 \pm 4.7^*$	3	14	17.0
			3	21	16.0
			3	35	19.0
					$18.5 \pm 1.7^*$
4	0	18.0	4	1	22.5
4	8	15.0	4	8	21.0
4	8	15.3	4	8	21.8
		$16.1 \pm 1.7^*$			$21.8 \pm 0.5^*$
5	0	28.4	5	1	25.6
5	0	27.0	5	1	27.9
5	7	33.0	5	7	24.0
		$29.3 \pm 3.7^*$			$25.9 \pm 2.0^*$

\* Mean and standard deviation.

or stimulation, as has been reported for the microbiological assays for some vitamins in blood.

*Recovery Experiments*—In order to determine the reliability of the method of protein precipitation and the assay procedure itself, recovery experiments were performed. The amino acids to be recovered were added in solution to the plasma previous to precipitation with sodium tungstate and the amount of water was reduced accordingly. In this way a 1:3 ratio between

TABLE III

*Free Amino Acid Content of Plasma Removed from Fasted Mongrel Dogs*

Dog No. and sex	Leucine	Isoleucine	Threonine	Valine
Samples removed under nembutal anesthesia				
	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.	$\gamma$ per cc.
1 ♂		10.5		21.5
2 ♀	14.7	9.5	30.0	17.7
3 ♀	14.8	9.5	35.0	18.5
4 ♂	18.9	13.7	16.1	21.8
5 ♂	22.4	14.9	29.3	25.9
6 ♀	22.7	18.0	31.5	24.6
Average and standard deviation.....	18.7 $\pm$ 3.9	12.7 $\pm$ 3.7	28.4 $\pm$ 7.3	21.7 $\pm$ 3.3
Samples removed without anesthesia				
7 ♂	16.4	8.8	29.4	15.2
8 ♂	22.0	12.2	28.4	20.9
9 ♂	30.0	17.1	15.0	30.0
10 ♂	30.6	19.5	28.7	30.3
11 ♀	14.3	8.7	12.3	15.2
12 ♂	22.9	15.3	27.6	25.0
Average and standard deviation.....	22.7 $\pm$ 6.8	13.6 $\pm$ 4.5	23.6 $\pm$ 7.4	22.8 $\pm$ 6.8
Recapitulation for all 12 dogs				
Range.....	14.3-30.6	8.7-19.5	15.0-35.0	15.2-30.3
Mean.....	20.9	13.1	25.8	22.2
Standard deviation..	5.6	3.8	7.3	5.3
“ error .....	1.7	1.1	2.2	1.7

plasma and filtrate was maintained. The amino acids were added in the *dl* form in twice the amount of *l* form required, since the *d* form was considered to have no activity. As may be seen from Table I, recoveries are equally satisfactory for leucine, isoleucine, valine, and threonine and indicate the quantitative nature of the procedures.

*Reproducibility of Assays and Effect of Storage of Plasma at -17°*—Plasma from the same dog was divided into several portions and kept at

-17° for periods up to 35 days. These portions were then thawed and analyzed for free amino acid content at different time intervals, as indicated in Table II. When 0 day of storage is shown, the filtrate was prepared from fresh plasma on the day it was removed from the dog. The data indicate that even after storage for 35 days at -17° values may be reproduced within 15 per cent of the mean, while samples of plasma assayed in duplicate at the same time check within 10 per cent.

*Amino Acid Content of Dog Plasma*—The results of the amino acid assay of plasma from twelve mongrel dogs are shown in Table III. For the purposes of our experiments it was important to know whether nembutal anesthesia had any effect on the free leucine, isoleucine, valine, or threonine content of the plasma. It is evident that while there appears to be some range in the values for each amino acid in both groups of dogs there is no significant effect of nembutal on amino acid concentration. Within the limits of accuracy that can be attained with the small number of observations, this may be tested by application of the formula  $(m_1 - m_2)/\sqrt{e_1^2 + e_2^2}$  where  $m_1$  and  $m_2$  are the two mean results and  $e_1$  and  $e_2$  are their respective standard errors. The values are as follows: leucine 1.29, isoleucine 0.38, valine 0.39, and threonine 1.66. According to Burn (6) a value of less than 2 indicates no significant difference between the groups.

The combined values for the amino acid content of plasma from anesthetized and unanesthetized fasted dogs are also given in Table III.

#### DISCUSSION

The data presented indicate the reliability and reproducibility of these methods for the determination of apparent free leucine, isoleucine, valine, and threonine in dog plasma. We have also found, in a limited number of samples, that the methods apply equally well to human and rabbit plasma. It is of interest that our attempts to measure free glutamic acid in dog plasma by a similar microbiological procedure were unsuccessful because of the low concentration of this amino acid. Our results showed that dog plasma contains less than 6  $\gamma$  of free glutamic acid per cc. in six samples examined. This was the limit of sensitivity of our method and the actual concentration may be considerably smaller. The low glutamic acid content of the plasma may indicate a low threshold for this amino acid. This may be related to the nausea and vomiting caused by intravenous injection of glutamic acid reported by Unna and Howe (7) and Madden (8). The comparatively large amount of glutamine present in dog plasma reported by Hamilton (9) was not measured by this procedure although glutamine does stimulate the growth of *Lactobacillus arabinosus* in the absence of glutamic acid. Lyman *et al.* (10) reported that most of the glutamine added to their assay tubes was converted to pyrrolidonecarboxylic acid when the tubes were autoclaved previous to inoculation. This substance does not

stimulate *Lactobacillus arabinosus*. Presumably, a similar conversion occurred with the dog plasma filtrate samples.

The mean values for leucine, isoleucine, valine, and threonine presented in Table III are typical of the mongrel dogs studied. We have not chosen to call them normal values, since the nutritive state of the animals was not controlled. It would be preferable to obtain normal values from dogs bred and raised under controlled laboratory conditions. However, these values are significant in showing concentrations of the free amino acids and their relative proportions in fasted dog plasma.

These methods probably have a fairly high degree of specificity. Hegsted (11) has shown that isoleucine is effectively replaced by the corresponding  $\alpha$ -hydroxy acid although leucine and valine are not, while of the acetyl derivatives only acetylleucine is active and all three  $\alpha$ -keto acid derivatives are somewhat active. The presence of any of these derivatives in blood filtrates remains to be proved. High levels of pyridoxal or pyridoxamine may lead to high threonine values, since the organism can utilize these to produce its own threonine (4, 12).

#### SUMMARY

Microbiological methods are presented for the determination of free leucine, isoleucine, valine, and threonine in dog plasma.

Plasma samples from twelve fasted mongrel dogs were found to contain 14.3 to 30.6  $\gamma$  per cc. of leucine with a mean value of  $20.9 \pm 5.6$ , 8.7 to 19.5  $\gamma$  per cc. of isoleucine with a mean value of  $13.1 \pm 3.8$ , 15.0 to 35.0  $\gamma$  per cc. of threonine with a mean value of  $25.8 \pm 7.3$ , and 15.2 to 30.3  $\gamma$  per cc. of valine with a mean value of  $22.2 \pm 5.3$ . Five samples of dog plasma were found to contain less than 6.0  $\gamma$  per cc. of glutamic acid. No differences in the free amino acid content of plasma were found in anesthetized animals as compared with unanesthetized animals.

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# FATE OF INGESTED SULFIDE SULFUR LABELED WITH RADIOACTIVE SULFUR IN THE RAT\*

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It is well known that in the metabolism of some sulfur-containing amino acids in the intestinal tract hydrogen sulfide may be formed (1). This product, due to its high coefficient of absorption (3), could be rapidly absorbed in large amounts if its formation were extensive. The rat, as well as man, may have sulfide sulfur generated in the liver from cysteine or cystine (12, 14). Another source of hydrogen sulfide, for some individuals at least, is the hydrogen sulfide which may contaminate the air of their environment. The question arises, therefore, as to the fate of the sulfide sulfur in the animal organism.

Haggard (9) demonstrated that oxygenated plasma as well as whole blood could oxidize sulfide sulfur. On the basis of his experiment on the repeated slow intravenous administration of sodium sulfide to a dog he concluded that the rate of oxidation in the intact animal may be such that many times the lethal amount can be handled without any apparent ill effect. Denis and Reed (5) obtained some evidence that sulfide sulfur may be oxidized to sulfate sulfur in the dog after intravenous injection of sodium sulfide. They further noted that after feeding powdered sulfur to dogs some 10 per cent of it appeared in the urine as sulfate sulfur and neutral sulfur (6). It was postulated that the elementary sulfur, which itself is insoluble in the intestinal juices, was converted by the intestinal flora to hydrogen sulfide and so absorbed, and that the hydrogen sulfide was oxidized to sulfate sulfur in the animal organism. Andrews has demonstrated that such a conversion of the elementary sulfur to sulfide sulfur may occur *in vitro* by the action of the intestinal flora of the dog (1).

The present report is concerned with the distribution of labeled sulfur in the organs and excretions of rats following the administration of sodium sulfide containing radioactive sulfur ( $S^{35}$ ).

## EXPERIMENTAL

A sample of barium sulfide containing radioactive sulfur ( $S^{35}$ ) was suspended in a small volume of distilled water and allowed to react with a slight excess of sodium sulfate for 48 hours at room temperature in a well

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stoppered tube. The resultant barium sulfate was filtered off and the filtrate used in these experiments.

White, male albino rats of approximately 5 months of age were placed in metabolism cages suspended over large funnels which were loosely stoppered with hobnailed glass balls to allow urine collection with a minimum contamination with feces. The animals were fed Purina dog chow in separate cages for 30 minutes at the commencement of each 24 hour period. Drinking water was allowed at all times.

Two rats of the same age and approximate body weight were each given 2 ml. of a solution of sodium sulfide containing  $S^{35}$  (approximately 1.66 mg. of sulfide sulfur) by stomach tube at the end of their daily eating period. The urine and feces were collected for the following 24 hours. At the end of this period the animals were anesthetized with nembutal, and the various tissues dissected out and weighed immediately. The hair was pulled from the skin and investigated separately.

The inorganic sulfate sulfur, total sulfate sulfur (8), and the total sulfur (4) of the urine were isolated as barium sulfate by centrifugation. The amount of the sulfate sulfur in the samples was adjusted to approximately the same concentration by the addition of 0.01 N sulfuric acid before precipitation with barium chloride. This was also done in the case of the sulfate sulfur obtained from the various tissues analyzed. When possible the tissues of these rats were analyzed for total sulfur by Bailey's (2) method. In other cases, an analysis of the sulfur content of tissues of normal rats was used as a basis for an estimation of the expected concentration.

Weighed amounts of the tissues and feces were hydrolyzed for 8 to 12 hours with 10 per cent sodium hydroxide on a steam bath. The hydrolysates were then transferred to nickel crucibles and oxidized with a mixture of sodium carbonate and sodium peroxide, as recommended by Bailey (2). The barium sulfate was precipitated from a solution of the melt after it was made acid to Congo red paper. The barium sulfate was isolated by centrifugation. Diluted samples of the stock sodium sulfide solution, containing the radioactive sulfur, were similarly treated.

After washing the barium sulfate precipitates twice with distilled water they were suspended in 70 per cent ethyl alcohol and transferred to shallow metal cups<sup>1</sup> in which a small drop of glycerol had been placed. (The glycerol was used as a binder to prevent loss of the barium sulfate when the latter was dried.) After complete transfer of the precipitate had been effected, it was resuspended in the cup by gentle stirring with a fine glass

<sup>1</sup> Tops or bottoms of seamless tin boxes ( $\frac{1}{2}$  ounce size) manufactured by the Buckeye Stamping Company, Columbus, Ohio, have been found to be convenient and inexpensive receptacles for the samples.

rod to promote an even distribution over the surface. The liquid was permitted to evaporate at room temperature and the cups were then dried in an oven at  $110^{\circ}$  overnight. The above preparation of barium sulfate samples for radioactivity determination is essentially that developed by Hendricks *et al.* (10).

The relative concentration of radioactive sulfur ( $S^{35}$ ) in the barium sulfate samples was determined with a Geiger-Müller, bell type counter (Massachusetts Institute of Technology design) in which the filling gas is a mixture of denatured alcohol and argon at a pressure of 39 cm. of mercury. The mica window has a thickness of  $10\ \mu$  (2.8 mg. per sq. cm.). The rate of disintegration was recorded with an Esterline-Angus graphic instrument, model A. W. All samples were counted on the same day to make them comparable without calculation of the different degrees of disintegration.

In a second experiment, two rats of approximately the same weight received 0.25 ml. each of a solution of sodium sulfide, containing radioactive sulfur (approximately 0.20 mg. of sulfide sulfur) by intraperitoneal injection immediately after their daily feeding period. Urine and feces were collected in 24 hour periods for the following 6 days. The urinary sulfur of the first 24 hour period was fractionated into inorganic sulfate sulfur, total sulfate sulfur, and total sulfur. All samples of feces and subsequent 24 hour urine samples were analyzed for total sulfur only.

On the 7th day after the injection of the sodium sulfide solution containing radioactive sulfur ( $S^{35}$ ), the same animals were each given 20 mg. of phenol (Merck, U. S. P., 98 to 100 per cent pure) in 2 ml. of distilled water by stomach tube. This was done immediately after their daily feeding period. 15 minutes later they were given 0.25 ml. each of the sodium sulfide solution containing  $S^{35}$  (approximately 0.20 mg. of sulfide sulfur) by intraperitoneal injection. The urine and feces were again collected for the following 24 hours. The urinary sulfur was fractionated.

#### DISCUSSION

The results obtained in the experiment in which sodium sulfide containing radioactive sulfur ( $S^{35}$ ) was administered by stomach tube are given in Table I. An examination of the data reveals that the sulfide sulfur was well absorbed and excreted in the urine. The major portion of the radioactive sulfur in the urine was found in the form of sulfate sulfur, both as inorganic sulfate sulfur and as ethereal sulfate sulfur. It would appear that the rat can oxidize sulfide sulfur to sulfate sulfur and thereby change a relatively toxic form of sulfur to one usually regarded as innocuous. This observation is in agreement with the results of Denis and Reed (5) obtained with dogs.

Whether the relatively high concentration of radioactive sulfur ( $S^{35}$ ) in

the feces and gastrointestinal tract is due to unabsorbed sulfide sulfur is not decided. Much of it may represent unabsorbed sulfide sulfur. Other possibilities, however, suggest themselves. Despite precautions to mini-

TABLE I

*Concentration of Radioactive Sulfur ( $S^{35}$ ) in Excreta and Tissues of Male Albino Rats 24 Hours after Oral Administration of Sodium Sulfide Containing Radioactive Sulfur ( $S^{35}$ )*

Each rat was given  $11.17 \times 10^6$  counts per minute of  $S^{35}$  (1.66 mg. of sulfide sulfur).

Tissue	Rat S1, 215 gm.				Rat S2, 208 gm			
	Tissue weight	Counts $\times 10^{-2}$ per gm. per min.	Counts $\times 10^{-4}$ per mg. S per min.	Counts recovered	Tissue weight	Counts $\times 10^{-2}$ per gm. per min.	Counts $\times 10^{-4}$ per mg S per min.	Counts recovered
	gm.			per cent	gm.			per cent
Urinary inorganic $SO_4$ sulfur	100*	458.5	50.94	41.0	100*	388.5	45.70	34.7
Urinary total $SO_4$ sulfur	100	548.5	54.85	49.1	100	538.5	53.85	48.2
Urinary total sulfur	100	877.0	73.08	78.5	100	577.0	52.45	51.7
Feces	0.71	6655.0	23.71	4.23	1.28	2020.0	7.52	2.31
Gastrointestinal tract	11.56	612.1	5.13	6.33	10.61	639.0	5.34	6.07
Bones	23.43†	114.3	1.43	2.40	22.67†	106.3	1.33	2.15
Heart	0.47	40.6	0.82	0.017	0.59	27.3	0.58	0.014
Kidneys	1.76	98.4	0.52	0.15	1.76	90.8	0.48	0.14
Spleen	0.31	112.8	0.42	0.03	0.31	90.2	0.34	0.02
Gonads	4.52	22.9	0.26	0.09	3.90	33.7	0.38	0.11
Lungs	0.82	69.2	0.34	0.05	0.94			
Blood	12.80†	39.3	0.21	0.45	12.36†	47.9	0.25	0.53
Liver	5.66	25.8	0.17	0.13	8.76	42.5	0.28	0.33
Brain	1.80	14.8	0.18	0.02	1.66	13.9	0.17	0.02
Skin	38.70†	42.4	0.53	1.47	37.44†	12.8	0.16	0.43
Muscle	97.61†	9.7	0.046	0.85	94.43†	7.6	0.036	0.64
Hair	4.17	1.5	0.0003	0.005	4.72	3.8	0.0008	0.016
Total	203.61			94.722	200.15			64.48

\* Urine diluted to 100 ml., the latter as an approximation assumed to weigh 100 gm.

† Donaldson ((7) p. 185).

‡ Calculated from the blood mass =  $0.09603$  (body weight)<sup>0.9</sup>  $\times 1.056$  ((7) p. 288).

mize the contact of urine and feces, it may be that part of the sulfur in the latter was due to contact with the urine.

The high concentration of the radioactive sulfur ( $S^{35}$ ) found in bone is interesting. Singher and Marinelli (11) observed that after the intra-peritoneal injection of sodium sulfate, in which the sulfur was labeled, the

highest concentration of the labeled sulfur was to be found in the bone marrow. It may well be that in the rats used in the experiment reported here a partial concentration of the radioactive sulfur ( $S^{35}$ ) in the bones occurred after the oxidation of sulfide sulfur to sulfate sulfur.

TABLE II

*Rate of Excretion of Radioactive Sulfur ( $S^{35}$ ) by Young Male Albino Rats after Intraperitoneal Injection of Sodium Sulfide Containing Radioactive Sulfur ( $S^{35}$ )*

Day	Sample	Rat S3			Rat S4		
		Counts $\times 10^{-3}$ per 24 hrs. per min.	Counts $\times 10^{-4}$ per mg. S per min.	Counts recovered	Counts $\times 10^{-3}$ per 24 hours per min.	Counts $\times 10^{-4}$ per mg. S per min.	Counts recovered
Each rat received $783.7 \times 10^3$ counts per min. $S^{35}$ (about 0.2 mg. sulfide sulfur)							
				per cent			per cent
1	Urinary inorganic $SO_4$ sulfur	4235.0	5.29	54.0	3885.0	6.47	49.6
	"    total $SO_4$ sulfur	4785.0	5.31	61.1	4785.0	6.38	61.1
	"    sulfur	5085.0	5.08	64.9	4985.0	5.54	63.6
	Feces	1277.0	1.03	16.3	1057.0	1.82	13.5
2	Urinary total sulfur	437.0	0.44	5.58	407.5	0.45	5.20
	Feces	109.7	0.11	1.40	151.7	0.14	1.93
3	Urinary total sulfur	120.0	0.12	1.53	136.0	0.15	1.75
	Feces	34.7	0.03	0.44	35.2	0.03	0.45
4	Urinary total sulfur	60.0	0.06	0.76	93.1	0.11	1.25
	Feces	11.7	0.01	0.15	17.2	0.02	0.22
5	Urinary total sulfur	52.8	0.05	0.67	120.0	0.12	1.53
	Feces	7.7	0.007	0.10	9.0	0.01	0.11
6	Urinary total sulfur	30.0	0.03	0.38	30.0	0.03	0.38
	Feces	2.9	0.003	0.04	5.3	0.005	0.06
Total 6 day excretion.....		7228.5		92.25	7052.0		89.95

Each rat received 20 mg. phenol orally 15 min. before intraperitoneal injection of sodium sulfide containing  $783.7 \times 10^3$  counts per min.  $S^{35}$  (about 0.2 mg. sulfide sulfur)

7	Urinary inorganic $SO_4$ sulfur	3085.0	10.28	39.4	3385.0	9.67	43.2
	"    total $SO_4$ sulfur	6235.0	5.66	79.5	5785.0	5.78	73.8
	"    sulfur	6385.0	4.91	81.5	5785.0	4.28	73.8
	Feces	1297.0	1.12	16.5	1277.0	1.17	16.3

Tarver and Schmidt (15) found that methionine sulfur may be used in the synthesis of cystine in the rat. On the other hand, sulfate sulfur was not used for this synthesis by rats. More recently, Smythe and Halliday (13) have shown the existence of an enzyme or enzyme system in livers of various animals, man and rat included, which is capable *in vitro* of con-

verting sulfide sulfur to cysteine sulfur. In view of this demonstration, it may be possible that the slight though definite concentration of radioactive sulfur ( $S^{35}$ ) found in the hair (Table I) is present as cystine sulfur. It may, however, have been deposited there as sulfate sulfur, since Singher and Marinelli (11) did detect some radioactive sulfur in hair of rats after the administration of sodium sulfate containing  $S^{35}$ .

Table II summarizes the results of the second experiment. These data again indicate the ability of the rat to oxidize sulfide sulfur to sulfate sulfur and to excrete the latter in part as ethereal sulfate sulfur. The concentration of radioactive sulfur ( $S^{35}$ ) in the feces was definite. However, it would be hazardous as yet to attempt to explain its presence there.

It is noteworthy that the major portion of the radioactive sulfur ( $S^{35}$ ) was rapidly excreted by the rats within 6 days. A large fraction appeared in the excreta within the first 24 hours and the amount then progressively decreased. Approximately 90 per cent of the radioactive sulfur ( $S^{35}$ ) was accounted for by the end of the 6th day following its administration as sulfide sulfur.

When the intraperitoneal injection of sodium sulfide containing radioactive sulfur ( $S^{35}$ ) followed the oral administration of phenol (Table II), the amount of radioactive sulfur ( $S^{35}$ ) recovered in the ethereal sulfate fraction was greater. Incidentally, it would seem that not all the phenol was excreted in combination with sulfuric acid. Some may have been excreted in combination with glucuronic acid, since the urines, when compared with urines collected from rats which had not received phenol, gave positive tests for glucuronic acid (the naphthoresorcinol test).

#### SUMMARY

About 50 per cent of the radioactive sulfur ( $S^{35}$ ) orally administered as sodium sulfide to rats appeared in the urine as sulfate sulfur within 24 hours. Part of it was present in the ethereal sulfate fraction. Small fractions of the administered radioactive sulfur were found in all the tissues examined, including the hair.

When sodium sulfide containing radioactive sulfur ( $S^{35}$ ) was intraperitoneally injected, the radioactive sulfur was excreted to the extent of approximately 90 per cent in the urine and feces in the course of 6 days. Some of it appeared in the urine as ethereal sulfate sulfur. The amount of radioactive sulfur ( $S^{35}$ ) appearing in the ethereal sulfate fraction was increased by the oral administration of phenol.

The interest and advice of Professor C. S. Robinson is gratefully acknowledged. The radioactive sulfur was kindly supplied by Dr. P. F. Hahn, who also graciously assisted with the radioactivity measurements.

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# ON THE ELECTROPHORETIC PATTERN OF RED BLOOD CELL PROTEINS\*

## A PRELIMINARY STUDY

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During the past 7 years, the electrophoretic behavior of serum and plasma proteins has been studied by numerous investigators (1, 2) and under a variety of conditions. In contrast to this situation in the field of *dissolved* blood proteins, the colloids present in the *formed* elements of blood seem to have received little or no attention from those engaged in electrophoresis research, except for a few isolated studies on purified hemoglobin (3). The bulk of experiments dealing with the electrochemical behavior of red blood cells have been performed on intact cells with the micro cataphoresis technique (4) and, hence, yield no information about the number and electrophoretic character of the intracellular proteins, since it can be shown that the behavior of a particle in an electrical field is a unique function of its surface (4).

The experiments, which are exploratory in nature, indicate that the electrophoretic patterns of hemolysates of *human*, *dog*, and *chicken* red blood cells are considerably less complex than those given by the plasma and serum of these species. The reason for this phenomenon is not the greater simplicity in composition of the erythrocyte compared with the surrounding medium but the fact that hemoglobin represents quantitatively by far the most dominant constituent of the non-nucleated red blood cell. Many of the other components are present in concentrations too small to give rise to boundaries detectable with the optical system here employed.

## EXPERIMENTAL

### *Materials and Technique*

The human red blood cells employed in this study were obtained from citrated whole blood withdrawn from normal or nearly normal subjects. In two instances, the red cell count was near the upper limit of the normal range or even indicated a slight degree of polycythemia (hematocrit cell volume readings, 50 and 50.7 per cent respectively). The dog and the

\* This work was made possible by a grant from the Carrie S. Scheuer Foundation of New York.



chicken<sup>1</sup> erythrocytes were separated from oxalated and citrated whole blood respectively, which, in the latter case, was pooled from several animals.

For the electrophoretic experiments, the cells were separated from the plasma by gentle centrifugation in an angle centrifuge. The top layer containing leucocytes was removed. The cells were repeatedly washed at the centrifuge with 0.9 per cent saline solution and then laked, in the packed state, by freezing in a dry ice-acetone bath and thawing at room temperature; this procedure was repeated twice. The laked cells were suspended in 4 or 9 volumes of the buffer to be employed in the electrophoresis experiment and then dialyzed against 2 liters of the same buffer (see below) for several days in the refrigerator. Prior to the experiments, the dialyzed solutions were centrifuged for 10 minutes at approximately 4000 R.P.M. in the angle centrifuge to remove suspended solids. In the case of the human and dog cells, the sediment thus obtained was negligible, indicating that almost all of the red blood cell contents had been brought into solution by the combined action of lysis and of the weakly alkaline buffer. In the instance of the red blood cells of the chicken, a bulky, gelatinous precipitate formed during the dialysis and was separated from the hemolysate by the subsequent centrifugation. This material probably represented nuclear proteins which are insoluble at pH 8.6, the pH chosen for the experiment. A hemolysate of rabbit red blood cells which was, however, not examined in the Tiselius apparatus, yielded a voluminous, colorless precipitate under similar conditions. Since rabbit red blood cells are non-nucleated, the nature of this precipitate remains to be determined.

In the majority of the experiments, the barbiturate buffer, recommended by Longworth (2) for human serum, was employed. This buffer contains 0.1 N sodium barbital and 0.02 N barbituric acid; its ionic strength is 0.1 and the pH 8.6. In one experiment with a human red blood cell hemolysate, a phosphate buffer mixture, 0.064 N with respect to  $\text{Na}_2\text{HPO}_4$  and 0.008 N with respect to  $\text{NaH}_2\text{PO}_4$ , of 0.2 ionic strength and pH 7.5 was used.

The electrophoresis experiments were carried out in a commercial Tiselius apparatus, with use of either the analytical cell constructed according to Tiselius and Longworth (*cf.* (2)) or the Tiselius separation cell of 11 cc. capacity. The temperature was held constant at 1.1°; the time of electrolysis was 12,000 to 17,000 seconds, with a current of 0.015 ampere and a corresponding potential gradient of  $F = 6.6$  volts per cm. The electrophoretic diagrams were visually observed with the Svensson crossed slit method and photographically recorded on Eastman panchromatic plates with Longworth's schlieren scanning technique; as light source a single filament tungsten lamp was used. This made possible the observation

<sup>1</sup> For the sample of chicken blood the authors are indebted to Mr. H. J. Noebels.

and recording of refractive index gradients arising in the deeply red-colored hemolysates during electrophoresis. The mobilities of the various components giving rise to maxima in the diagrams were computed by measuring directly on the original negatives the distances of the peaks from the starting position of the boundary and dividing this distance (in cm.) by the photographic magnification factor of the camera (1.094), the time of electrolysis (in seconds), and the potential gradient (in volts per cm.). The mobilities were determined both for the descending and the ascending limb of the apparatus. The pH and conductivity of the various solutions were determined with the glass electrode and a conductivity bridge respectively.

The analytical determinations, performed on the original solutions and on some of the electrophoretic fractions, included nitrogen (micro-Kjeldahl), protein by Wu's tyrosine method, and hemoglobin by photoelectric colorimetry, checked by determining the oxygen capacity of the blood (for details of the analytical procedures see *e.g.* Reiner (5)).

### *Observations and Results*

The most striking feature of the electrophoretic behavior of all red blood cell hemolysates examined thus far is the separation, in the ascending limb of the cell, of an *opalescent, colorless* component with an anodic mobility about twice as high as that of oxyhemoglobin at the pH values selected for these preliminary experiments. The phenomenon is best observed by direct inspection of the electrophoresis cell, either in transmitted or in incident light. In some experiments, the boundary of the opalescent material exhibited a cusped appearance and in others a slight amount of streaming, reminiscent of the behavior of dilute suspensions of elementary bodies of vaccinia during electrophoresis (6). In the descending limb of the cell, the relatively fast migration of the trailing boundary of the opalescent component leaves behind purified hemoglobin, resulting in a distinctly enhanced transparency of the deep red solution. The effect may best be compared with the removal of a veil or shadow from an underlying brightly colored object. Owing to the limitations imposed upon the schlieren method by strongly colored and turbid media, the schlieren scanning photographs fail to bring out the features just mentioned in their full distinction.

Under the experimental conditions employed, the ascending boundary of the opalescent component reached the top of the anode limb of the Tiselius-Longsworth analytical cell at a time when the oxyhemoglobin boundary had progressed to about the middle of the compartment. On the cathode side, the downward movement of the opalescent material and the slower migration of the hemoglobin boundary left a clear and colorless zone in the upper part of the cell, which, as far as can be judged, did not contain a colloidal component. The stationary peak recorded in that limb would

thus correspond to the buffer-buffer concentration anomaly which, in the case of blood serum, has been called the  $\epsilon$ -boundary (1, 2). A corresponding stationary peak, designated as the  $\delta$ -boundary, was observed and recorded in the ascending limb of the cell.

Typical schlieren scanning diagrams, obtained with *human* hemolysates, are reproduced in Fig. 1. In these diagrams, there are recorded the low peak due to the opalescent, colorless material (*a* component), the dominant peak of the oxyhemoglobin (Hb), and a very small maximum due to a colorless component (*b* protein) of a mobility lower than that of hemoglobin, in addition to the  $\delta$  and  $\epsilon$  anomalies. The *a* and the *b* components amount to only a few per cent of the total protein present. A quantitative estima-

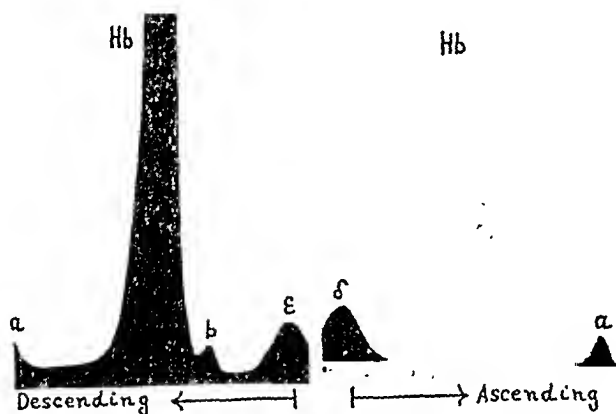


FIG. 1. Electrophoretic patterns obtained with *human* red blood cell hemolysates. The diagrams of the descending and ascending boundaries were obtained in different experiments, with different samples of human erythrocytes. They are paired here merely for the purpose of illustrating the phenomena discussed in the text.

tion of their concentration from the diagrams by planimetry is not possible because the experiments had to be conducted at hemoglobin concentrations too high to record the maximum of the hemoglobin peak on the plate by the scanning method. Moreover, the refractive increments of these components are not as yet known. Protein determinations by Wu's tyrosine method and micro-Kjeldahl determinations on the various fractions, removed from the cell after electrophoresis, indicated that the opalescent *a* component amounts to only 2 to 5 per cent of the hemoglobin concentration. The Kjeldahl determinations are complicated by the nitrogen content of barbiturate. In one experiment with phosphate buffer, the nitrogen content of the compartments containing the hemoglobin was about 6 mg. per cc., whereas the corresponding figure for the *a* fraction was 0.16 mg. per cc. The *a* fraction may readily be obtained free from contamination by

hemoglobin; on the other hand, the hemoglobin fraction is contaminated by the *b* protein which, at this pH, migrates at a rate so similar to that

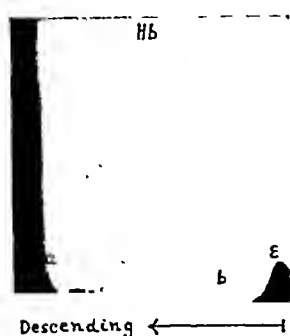


FIG. 2

FIG. 2. Electrophoretic diagram, recording the descending boundaries of a *dog* red blood cell hemolysate. (See also Table I.)

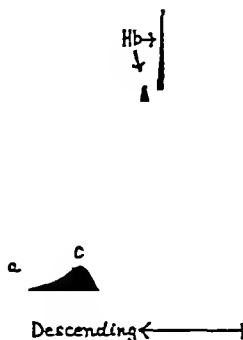


FIG. 3

FIG. 3. Pattern, recording the descending boundaries of a *chicken* hemolysate. (See Table I.)

TABLE I

*Electrophoretic Mobilities of Components of Red Blood Cell Hemolysates\**

Experimental conditions, barbiturate buffer, pH 8.6,  $\mu = 0.1$ ; potential gradient,  $F = 6.64$  volts per cm.; temperature,  $1.1^\circ$ .

Experiment No.	Red cells, species	Duration of electrolysis <i>sec.</i>	Limb of cell	Mobilities of components $\times 10^5$			
				<i>a</i> protein	Hemoglobin	<i>b</i> protein	<i>c</i> protein
371	Human	12,900	Ascending	5.81	2.91	1.48	†
374	"	14,400	"	7.22	†	†	†
			Descending	7.51	3.50	2.24	
376	Dog	12,900	Ascending	6.14	2.57	†	†
			Descending	6.43	2.57	1.59	
384	Chicken	16,800	Ascending	†	4.00, 2.91	†	
			Descending	†	3.50, 2.79	†	5.60

\* The assistance rendered by Mr. S. Singer in the calculation of the mobilities listed in this table is gratefully acknowledged.

† Not present.

‡ Not determined.

of hemoglobin that the compensation principle of Tiselius would have to be employed to effect a significant separation of these two components.

The diagrams recorded upon electrophoresis of *dog* and *chicken* hemolysates are reproduced in Figs. 2 and 3 respectively.

It will be noted that the diagram obtained with dog erythrocytes (Fig. 2) is very similar to those obtained with human red blood cells (Fig. 1). The chicken hemolysate, on the other hand, yields a pattern (Fig. 3) which differs greatly from those recorded for the two mammalian species: The hemoglobin boundary, in the descending limb, shows a distinct split into two components of similar mobility and, furthermore, there is present in the same limb a colorless component (*c* protein), of a mobility intermediate between that of the opalescent component (*a* protein) and the two hemoglobin fractions. There is no evidence in the avian red blood cell diagram for the presence of the *b* protein encountered in human and dog cell hemolysates.

The mobilities of the various boundaries observed in the hemolysates are listed in Table I.

#### DISCUSSION

Analytical work on the composition of red blood cells (7) has shown that, in non-nucleated erythrocytes, hemoglobin accounts for 87 to 95 per cent of the total solids. In addition, 5 to 12 per cent of other proteins, about 0.6 per cent lecithin, about 0.4 per cent cholesterol, and a number of inorganic constituents have been found by chemical methods. Nucleated red blood cells, on the other hand, may contain as much of other proteins as they do of hemoglobin. In addition, erythrocytes are known to contain small amounts of sugars, glucuronic acid, creatine, and hemocuprein (a copper protein), as well as the full complement of enzymatic and non-enzymatic catalysts required for glycolysis and, in the instance of the nucleated cells, also for respiration.

The low molecular constituents are removed by the dialysis preceding the electrophoresis. The majority of the colloids contained in the red blood cell, because of their low absolute and relative concentration, do not appear in the electrophoretic diagrams as obtained with hemolysates under our experimental conditions. With the exception of hemoglobin, the location of which in the electrophoresis cell may be readily ascertained with the aid of its color and spectrum, the nature of the other electrophoretic components (*a*, *b*, and *c* protein) here observed and their possible identity with known red blood cell constituents remain to be established.

Among the components here observed, the greatest interest attaches to the opalescent, colorless material of high anodic mobility at pH 8.6 (*a* component). The Tyndall effect given by this fraction indicates that the individual particles possess an appreciable size, but it cannot be decided at this time whether one is dealing here with a macromolecular protein (or lipoprotein) or with structural cell fragments in suspension. If this material is derived from the red blood cell membrane or the stroma, which

may be identical or closely related in their composition,<sup>2</sup> its electrophoretic mobility should agree with the mobility of intact erythrocytes as determined with the microscopic cataphoresis technique. The mobility of human red blood cells at pH 8.35 and an ionic strength of 0.172, expressed in terms of  $V$  ( $\mu$  per second per volt) is given as 1.06 at 25° (8). Upon conversion into  $u$  ( $\text{cm}^2$  per second per volt), the mobility unit employed in moving boundary experiments, this value becomes  $10 \times 10^{-5}$ . In order to reduce this value to the mobility at 1°, it must be divided by a factor of 2. The resulting figure, *viz.*  $u = 5 \times 10^{-5}$ , is in satisfactory agreement with the values found for the mobility of the opalescent *a* protein in the present experiments (see Table I), thus lending strong support to the assumption that this component is contained in the surface of the red blood cell. It is planned to investigate this point further by chemical comparison of purified stroma protein with *a* protein prepared by electrophoresis.

It is planned to extend this investigation to cover a wider pH range, as well as to other animal species and to hemolysates obtained by various procedures from *normal* as well as *pathological* blood cells.

#### SUMMARY

Preliminary experiments on hemolysates from several species (man, dog, and chicken) by the moving boundary method of electrophoresis have disclosed patterns considerably less complex than those recorded for the serum and plasma of the same species. The major component in the erythrocyte diagrams is represented by hemoglobin, as is to be expected from the analytical composition of the red blood cell. In addition, all hemolysates examined showed the presence of a small amount of an opalescent, colorless fraction (designated as *a* protein) of an anodic mobility of about  $6 \times 10^{-5} \text{ cm}^2$  per volt per second at pH 8.6, as compared to about  $3 \times 10^{-5}$  for the hemoglobin. The mobility of the opalescent material is of the same order as that of intact erythrocytes, suggesting that it is derived from the membrane or the stroma of the cells. In addition to these components, human and dog hemolysates contain a minor, colorless component (*b* protein) of a mobility somewhat smaller than that of hemoglobin, while chicken red blood cells possess, instead, a component (*c* protein) of a mobility intermediate between that of hemoglobin and the opalescent material (*a* protein).

The identity of the various components other than hemoglobin remains to be established.

<sup>2</sup> Ponder, E., and Furchgott, R., personal communication.

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# ITATARTARIC ACID, A METABOLIC PRODUCT OF AN ULTRA-VIOLET-INDUCED MUTANT OF *ASPERGILLUS TERREUS*

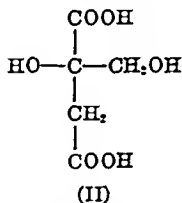
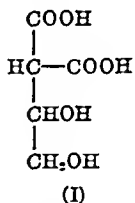
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(Received for publication, October 18, 1945)

A survey (1, 2) of numerous strains of *Aspergillus terreus* to determine their ability to convert sugars to itaconic acid has shown that, in addition to this unsaturated acid, some of the strains produce considerable amounts of saturated acids as well. The products of one of these strains, an ultra-violet-induced mutant, have been studied with the view of determining the nature of the acids.

For separation of the saturated acids, the methyl esters were fractionated *in vacuo* to yield two main fractions boiling at 129–134° and 151–154° at 2 to 3 mm. The lower boiling material was converted into an optically active benzylamide (m.p. 103°) showing the composition  $C_{11}H_{22}O_4N_2$ .



Cleavage of this derivative with periodic acid yielded formaldehyde and an optically inactive compound,  $C_{18}H_{18}O_2N_2$ , suggesting the parent acid to be a dihydroxylated dicarboxylic acid of the molecular formula  $C_5H_8O_6$ . Of the nine acids of this formula theoretically possible only two, I and II, can give formaldehyde on periodic acid cleavage. Since a compound with formula II is related to itaconic acid, it was considered the more likely possibility. To conform with this formula, the structure of the  $C_{18}H_{18}O_3N_2$

cleavage product would have to be  $C_6H_5-CH_2-NH-\overset{\overset{O}{||}}{C}-\overset{\overset{O}{||}}{C}-CH_2-\overset{\overset{O}{||}}{C}-NHCH_2-C_6H_5$ . That this is the correct structure was established by its reduction to a compound found to be identical with the dibenzylamide of *dl*-malic acid.

\* One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.



So far as we know, this is the first report of the formation of this homologue of tartaric acid by a microorganism. It has, however, been prepared synthetically (3-6) in the racemic form from itaconic acid and is known as itatartaric acid. Apparently, it exists only in the form of its salts. The lactone (6) of racemic itatartaric acid has, however, been isolated as a crystalline solid with a melting point of  $104^{\circ}$ . Our ester fraction with a boiling point of  $151-154^{\circ}$  is presumably the methyl ester of the lactone, since it gives the same benzylamide as the lower boiling fraction. The lactone methyl ester fraction (6.58 gm.) corresponds to 5.62 gm. when converted to the dibasic acid; similarly, the lower boiling fraction (5.40 gm.) is equivalent to 4.61 gm. of the same acid. The yield of itatartaric acid, then, is 10.23 gm. or 5.8 per cent of the total weight of acid produced in the fermentation.

#### EXPERIMENTAL

*Fractionation of Methyl Esters*—The strain of *Aspergillus terreus* used (Northern Regional Research Laboratory, No. 265.S14) is an ultraviolet-induced mutant (7) of an organism isolated originally from Texas soil and carried in the laboratory collection as Northern Regional Research Laboratory, No. 265. It was grown at  $30^{\circ}$  for 10 days in 3 liter flasks (750 cc. per flask) on a medium of the following composition: Glucose 220 gm.,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.50 gm., KCl 0.05 gm.,  $\text{H}_3\text{PO}_4$  0.418 gm.,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  0.022 gm., 0.50 N HCl 4.0 cc., 0.50 N  $\text{HNO}_3$  8.0 cc.,  $\text{NH}_4\text{NO}_3$  4.0 gm., corn steep liquor 1.0 gm., and distilled water to 1 liter.

The culture liquor, after removal of the mold mats, was concentrated *in vacuo* (bath  $50^{\circ}$ ) from a volume of 3.75 liters to 300 cc. The crystals of itaconic acid separated during cooling in the ice box were filtered off. The filtrate was then extracted continuously with ether for 24 hours. The ether extract was concentrated several times to remove more itaconic acid. A total of 134.9 gm. of crystalline itaconic acid was obtained from the 3.75 liters of culture liquor. The filtrate, freed of most of the itaconic acid, was then concentrated to a gum weighing 43.8 gm. This residue was dissolved in methanol and an excess of diazomethane in ether added. Repeated fractionation of the methyl esters with a Vigreux column gave the following main fractions:  $83-95^{\circ}$  (5 mm.), weight 6.31 gm. (mostly methyl itaconate as judged by the boiling point and bromine absorption);  $95-105^{\circ}$  (3 mm.), weight 2.74 gm.;  $129-134^{\circ}$  (2 to 3 mm.), weight 5.40 gm.;  $151-154^{\circ}$  (2 to 3 mm.), weight 6.58 gm.; and a tarry residue.

*Benzylamide from Fraction with Boiling Point of  $129-134^{\circ}$* —This methyl ester fraction with a boiling point of  $129-134^{\circ}$  was a viscous, pale yellow oil which on analysis proved to be  $\text{C}_{7.26}\text{H}_{11.6}\text{O}_6$  and showed an  $[\alpha]_D^{23} = -29^{\circ}$ . 700 mg. were heated for 2 hours at  $60^{\circ}$  with 0.5 cc. of methanol and 2.0 cc.

of benzylamine. The reaction product was dissolved in ether and the solution washed with dilute hydrochloric acid. The crystals (675 mg.) obtained by concentration of the ether were crystallized from methanol-ether in the form of long needles melting at 103–104°. Tests for sulfur, carbonyl, and alkoxy were negative. The rotation was  $[\alpha]_D^{25} = -42^\circ$  (55.1 mg. in 1.50 cc. of 95 per cent alcohol).

$C_{11}H_{11}O_4N_2$ .	Calculated.	C 66.49, H 6.48, N 8.18
	Found.	" 66.6, " 6.18, " 8.30 (Kjeldahl)
		" 66.8, " 6.39, " 8.16 "

*Periodic Acid Cleavage*—To the benzylamide (192 mg.), dissolved in 7.5 cc. of methanol, was added a solution of 0.5 gm. of  $H_6IO_6$  in 0.5 cc. of 1 N  $H_2SO_4$ . Needles appeared in about a minute and were filtered off 1 hour later after the addition of 2 volumes of cold water. The product (115 mg.) melted at 172–173° with evolution of gas. Two crystallizations from acetone did not raise the melting point. The product is optically inactive. In alcohol solution, it gives a deep reddish color with  $FeCl_3$ .

$C_{11}H_{11}O_4N_2$ .	Calculated.	C 69.66, H 5.85, N 9.03
	Found.	" 69.6, " 5.83, " 8.94 (Kjeldahl)
		" 69.5, " 6.02 "

The other cleavage product was identified as formaldehyde in the following manner. The benzylamide (9 mg.) was dissolved in 0.2 cc. of alcohol and 20 mg. of  $H_6IO_6$  in several drops of alcohol added. After 1 hour, the reaction mixture was diluted with an equal volume of water and the crystals removed by filtration. The filtrate was made slightly alkaline to litmus with  $K_2CO_3$  and then slightly acid with acetic acid. The precipitated  $KIO_4$  was filtered off and 12 mg. of dimethyldihydroresorcinol in 0.15 cc. of alcohol were added. The crystals were separated and crystallized from dilute alcohol, yielding 1.8 mg. of needles (m.p. 187–188°) showing no depression on melting with an authentic sample of the formaldehyde dimedon condensation product. The x-ray diffraction patterns were also found to be identical.

*Reduction of Periodic Acid Cleavage Product*—10 mg. of the cleavage product (m.p. 172–173°) were dissolved in 2 cc. of 1 N NaOH containing a few drops of alcohol. To this solution 0.6 gm. of 2 per cent Hg-Na was added gradually. Crystals appeared and were filtered off. They weighed 3 mg. and gave no color with  $FeCl_3$  in aqueous alcohol. Crystallization from alcohol gave 1.8 mg. of product melting at 145–146°. Mixed melting point test and x-ray diffraction patterns showed it to be identical with the dibenzylamide of *dl*-malic acid.

*dl-Malic Acid Dibenzylamide*—200 mg. of *dl*-malic acid in methanol were methylated with excess ethereal diazomethane. The methanol was re-

moved and the residue heated for 2 hours at 80° with 1 cc. of benzylamine. The reaction product was worked with ether and the resulting crystals removed by filtration. Crystallization from alcohol gave 220 mg. of the pure benzylamide in the form of bars melting at 145–146°.

$C_{18}H_{20}O_3N_2$ .	Calculated.	C 69.21,	H 6.45,	N 8.97
	Found.	" 69.4,	" 6.36,	" 8.60
				" 8.84

*Benzylamide from Fraction with Boiling Point of 151–154°*—The methyl ester fraction with a boiling point of 151–154° (2 to 3 mm.) was a viscous yellow oil showing 20.4 per cent methoxyl. The calculated value for the lactone methyl ester ( $C_8H_8O_6$ ) is 21.0 per cent. A sample of the methyl ester (135 mg.) was converted to the benzylamide with a melting point of 102–103° (106 mg.). There was no depression in melting point on admixture with the benzylamide from the lower boiling fraction.

#### SUMMARY

About 5.8 per cent of the total acids produced by an ultraviolet-induced mutant of *Aspergillus terreus* has been shown to be an equilibrium mixture of itatartaric acid and its lactone. These compounds have not previously been found to occur naturally.

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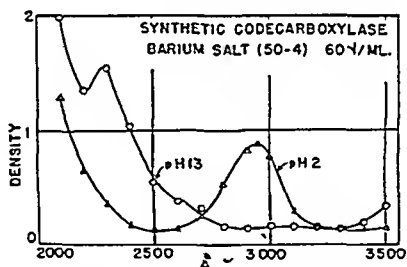
## LETTERS TO THE EDITORS

### SOME PROPERTIES OF SYNTHETIC CODECARBOXYLASE

Sirs:

Codecarboxylase<sup>1</sup> (phosphorylated pyridoxal<sup>2</sup>), the coenzyme of amino acid decarboxylases<sup>1, 3</sup> and of transaminase,<sup>4</sup> has now been synthesized and purified as the barium salt. The chemical properties and their correlation with coenzyme activity are also reported.

The treatment of pyridoxal<sup>5</sup> in aqueous solution with  $\text{POCl}_3$  has yielded codecarboxylase, which has been purified by the methods used for the frac-



tation of phosphate esters, the codecarboxylase being found in the water-soluble, alcohol-insoluble fraction of the barium salts. The yield of coenzyme was followed with the tyrosine decarboxylase assay<sup>6</sup> until yields were sufficient for purification.

<sup>1</sup> Gale, E. F., and Epps, H. M. R., *Biochem. J.*, **38**, 250 (1944).

<sup>2</sup> Gunsalus, I. C., Bellamy, W. D., and Umbreit, W. W., *J. Biol. Chem.*, **155**, 685 (1944).

<sup>3</sup> Gale, E. F., and Braddily, J., *Nature*, **155**, 727 (1945). Umbreit, W. W., and Gunsalus, I. C., *J. Biol. Chem.*, **159**, 333 (1945).

<sup>4</sup> Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, **161**, 311 (1945).

<sup>5</sup> We are indebted to Merck and Company, Inc., for the pyridoxal used in these experiments.

<sup>6</sup> Umbreit, W. W., Bellamy, W. D., and Gunsalus, I. C., *Arch. Biochem.*, **7**, 185 (1945).

The absorption spectrum of a sample prepared by this method, as given in the figure, differs from that of pyridoxal largely in the lack of an absorption band at 3000 Å in alkaline solution, the absorption spectrum of pyridoxal in the ultraviolet being similar to that of pyridoxine.<sup>7</sup> Synthetic codecarboxylase does not give a phenol reaction by the chloroimide method,<sup>8</sup> which is positive for pyridoxal.

The sample contains 6.2 per cent organic phosphorus, 32 per cent pyridoxal by spectrum (1 atom of phosphorus per mole of pyridoxal), and about 50 per cent barium. The coenzyme activity is about 18 times that of the

*Rate of Hydrolysis of Synthetic Codecarboxylase at 100° in N HCl*

Time	Coenzyme destroyed	P liberated	Phenolic reaction liberated	Spectrum 3000 Å, pH 13
<i>min.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0	0	0	0	0
30	49	42	42	48
60	76	68	70	68
90	79	78	81	81
120	88	91	94	90
180	99+	99.7	100	100

Pyridoxal content of sample estimated from spectrum = 32 per cent; from chloroimide phenolic reaction after hydrolysis = 36 per cent.

pyridoxal-ATP standard used in the assay system, thus affording confirmation of data on codecarboxylase formation from members of the vitamin B<sub>6</sub> group by growing cells.<sup>9</sup>

Upon hydrolysis, in N HCl at 100°, the codecarboxylase activity is destroyed, as is indicated in the table, with a corresponding release of inorganic phosphate, appearance of a phenolic reaction, and increase in absorption at 3000 Å in alkaline solution.

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<sup>7</sup> Harris, S. A., Webb, T. J., and Folkers, K., *J. Am. Chem. Soc.*, **62**, 3198 (1940).

<sup>8</sup> Scudi, J. V., *J. Biol. Chem.*, **139**, 707 (1941).

<sup>9</sup> Bellamy, W. D., Umbreit, W. W., and Gunsalus, I. C., *J. Biol. Chem.*, **160**, 461 (1945).

# INTERMEDIATES OF ACETATE OXIDATION IN KIDNEY

Sirs:

The recent report of Buchanan *et al.*<sup>1</sup> disclosing that components of the Krebs cycle are major intermediates of acetate and acetoacetate oxidation in kidney substantiates previous conclusions of Breusch<sup>2</sup> and Wieland and Rosenthal.<sup>3</sup> A paradoxical feature, however, is that, whereas the conclusions of Breusch and Wieland are based on the actual formation of citric acid from acetoacetate and oxalacetate (confirmed by Hunter and Leloir<sup>4</sup>), the isotopic tracer study by Buchanan *et al.* appears to eliminate citric acid as a direct participant in acetoacetate oxidation.

*C<sup>13</sup> Concentration in Possible Intermediates of Acetate Oxidation in Kidney*

	Additions					
	Na citrate, 0.64 mM		Na cis-aconitate, 0.45 mM		Na α-ketoglutarate, 0.68 mM	
	mM	C <sup>13</sup> excess	mM	C <sup>13</sup> excess	mM	C <sup>13</sup> excess
Acetate utilized .	0.31	3.90	0.16	3.90	0.18	3.90
Intermediate recovered.	0.36	0	0.26 (As citrate)	0	0.38	0.24
Respiratory CO <sub>2</sub>	1.23	0.86	0.78	0.71	0.78	0.54

*Distribution of Excess C<sup>13</sup> in Ketoglutarate*

	Ketoglutarate	COOH adjacent to CO	Succinate	Succinate carboxyls
C <sup>13</sup> excess, atoms %	0.24	0.20	0.21	0.39

Using sodium acetate, labeled with C<sup>13</sup> in the carboxyl carbon, we have found, in agreement with Buchanan *et al.*, that acetate is converted to ketoglutarate by kidney slices. However, neither citrate nor substances in equilibrium with it appear to be intermediates of acetate oxidation in this organ.

When labeled acetate, together with non-isotopic α-ketoglutarate, was incubated aerobically with rat kidney mince, the recovered ketoglutarate (isolated as the semicarbazone, m.p. 200–202°) had a significant excess of

<sup>1</sup> Buchanan, J. M., Sakami, W., Gurin, S., and Wilson, D. W., *J. Biol. Chem.*, **169**, 695 (1945)

<sup>2</sup> Breusch, F. L., *Science*, **97**, 490 (1943).

<sup>3</sup> Wieland, H., and Rosenthal, C., *Ann. Chem.*, **554**, 241 (1943)

<sup>4</sup> Hunter, F. E., and Leloir, L. F., *J. Biol. Chem.*, **169**, 295 (1945).

C<sup>13</sup>. Permanganate oxidation and subsequent degradation of the resulting succinate by pyrolysis of the barium salt at 500° indicated that about one-sixth of the excess isotope was in the ketoglutarate carboxyl adjacent to the CO group, the remainder being in the carboxyl carbons of the succinate moiety.

When citrate and isotopic acetate were incubated with kidney mince, the recovered citrate (isolated as the trisodium salt) contained no excess C<sup>13</sup>. Repetition of this experiment with kidney and liver slices gave similar negative results. When isotopic acetate and *cis*-aconitate were incubated together under the same conditions, rapid establishment of aconitase equilibrium resulted in conversion of the unutilized aconitate to citrate, which again contained no measurable C<sup>13</sup> excess.

Negative results in such experiments must be interpreted with caution, inasmuch as it is possible that a biologically formed, isotopic intermediate may not come into equilibrium with the added substrate. However, in view of the facts (a) that acetate oxidation had doubtless occurred, as shown by the high isotope content of the respiratory CO<sub>2</sub>, and (b) that this same procedure was successful in demonstrating the conversion of acetate to ketoglutarate and to acetoacetate,<sup>5</sup> the conclusion seems warranted that citric acid as such is not in the direct pathway of acetate oxidation in kidney.

Inasmuch as aconitase was active, as was demonstrated by conversion of *cis*-aconitate to citrate, it would appear either that *cis*-aconitate is not an intermediate of acetate oxidation or that under the conditions of its biological formation it does not come into equilibrium with citric acid.

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<sup>5</sup> Medes, G., Weinhouse, S., and Floyd, N. F., *J. Biol. Chem.*, 157, 751 (1945).

# A NUTRITIONALLY PRODUCED CEREBELLAR DISORDER IN CHICKS, NOT ASSOCIATED WITH E AVITAMINOSIS

Sirs:

A disorder in chicks involving an incoordination of movements and convulsions was observed on highly purified diets of the following composition per 100 gm.: water-washed casein 22.2, *l*(+)-arginine monohydrochloride 0.3, glycine 0.9, *l*(-)-cystine 0.4, calcium gluconate 5.0, cellulose (Cellu flour) 5.0, soy bean oil 3.0, fish oil (sardine) (3000 A-400 D) 0.25, sodium chloride mixture containing 0.49 per cent manganese, 0.1 per cent copper, 0.05 per cent zinc, 0.05 per cent aluminum, 0.002 per cent cobalt, and 0.04 per cent iodine 1.0,  $\text{Ca}_3(\text{PO}_4)_2$  3.5,  $\text{K}_2\text{HPO}_4$  1.3,  $\text{KCl}$  0.3,  $\text{MgSO}_4$  0.1,  $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$  0.25, cholic acid 0.1, choline chloride 0.2, and glucose (cerelose) 56.2 gm. To every 100 gm. of the above mixture were added solubilized liver eluate

Day-old chicks in groups of 10	Average gain in weight in 14 days	Per cent of chicks with brain lesions	Per cent of chicks with gross symptoms
Purified ration + 0 mg. $\gamma$ -vitamin E* . . .	15	100	100
" " + 1 " " " " . . .	17	100	100
" " + 14 " " " " . . .	22	100	100
Commercial type ration	41		

\* Synthetic  $\alpha$ -tocopherol (Merck).

equivalent to 4 gm. of solubilized liver, thiamine hydrochloride 0.5 mg., pyridoxine hydrochloride 0.4 mg., riboflavin 0.5 mg., *d*-calcium pantothenate 1.5 mg., nicotinic acid 1.0 mg., 2-methyl-1,4-naphthohydroquinone diacetate 1.0 mg., synthetic  $\alpha$ -tocopherol (Merck) 1.0 mg., and biotin<sup>1</sup> 0.01 mg.

The solubilized liver eluate was prepared by extracting 1 kilo of solubilized liver<sup>2</sup> four times with 2 gallon portions of water brought to pH 4 with sulfuric acid. The solids were allowed to separate by gravity and the supernatant fluid was removed by siphon. The clear extract was then treated twice with 250 gm. portions of charcoal at pH 4. The charcoal was removed by filtration and eluted with a solution of 60 per cent acetone and 40 per cent water containing 2 per cent  $\text{NH}_4\text{OH}$ . The removal of the acetone and ammonia and the concentration of the eluate were effected by distillation *in vacuo*.

<sup>1</sup> Crystalline biotin donated by Merck and Company, Inc.

<sup>2</sup> Liver fraction L donated by The Wilson Laboratories, Chicago, Illinois.



The disorder was found to be associated with a cerebellar lesion, observable grossly in severely affected chicks and only microscopically in mild cases. While gross symptoms appeared in 70 different groups of chicks receiving the purified ration, none have been observed in chicks fed a commercial type ration composed of mixed natural feedstuffs. All chicks, affected by this disorder, whose brains were examined histologically, showed the cerebellar lesion. A full description of the syndrome and its production will be given in a later communication. Illustrative data are presented in the table, which shows that vitamin E in generous amounts does not prevent this disorder. The factor in natural foods protecting against these lesions has not thus far been identified, and is being further investigated in this laboratory.

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## INFLUENCE OF GELATIN AND TRYPTOPHANE ON NICOTINIC ACID REQUIREMENT OF CHICKS\*

Sirs:

Krehl *et al.*<sup>1</sup> recently reported that either tryptophane or nicotinic acid counteracts growth retardation, in rats, caused by the inclusion of corn grits in a low protein ration. This observation has been extended to the chick and, in addition, it has been found that tryptophane may successfully replace nicotinic acid in a purified diet not containing corn. Further work has shown that the presence of gelatin in the ration greatly increases the nicotinic acid requirement.

The basal diet, low in a source of arginine and glycine and in nicotinic acid,<sup>2</sup> consisted of glucose (cerelose) 68.4, casein 18, Salts 1M<sup>3</sup> 6, soy bean oil 4, liver fraction L 3, and *l*(-)-cystine 0.3. Each 100 gm. of diet contained the following amounts (in mg.) of vitamins: thiamine hydrochloride 0.4, riboflavin 0.8, Ca pantothenate 2.0, choline chloride 200, pyridoxine hydrochloride 0.6, biotin 0.02, *i*-inositol 100, *p*-aminobenzoic acid 0.2, 2-methyl-1,4-naphthoquinone 0.1, and  $\alpha$ -tocopherol 0.5. Vitamin A (1200 I. U.) and vitamin D (170 A. O. A. C. units) were supplied by dropper weekly. Substitutions in the ration were made at the expense of glucose. Day-old New Hampshire chicks were used throughout.

The feeding of 10 per cent of gelatin alone, as a source of arginine and glycine, caused a marked depression of the growth rate (compare Groups 1 and 5) and caused the production of typical nicotinic acid deficiency symptoms.<sup>2</sup> This inhibitory effect was not noted when gelatin was replaced by arginine and glycine, although the growth rate was probably not quite optimum.

All inhibitory action was counteracted by supplementing the gelatin with either 5 mg. of nicotinic acid or with 200 mg. of *dl*-tryptophane per 100 gm. of diet. Likewise, the addition of either nicotinic acid or tryptophane to the diet containing arginine and glycine produced similar rapid growth.

\* Scientific paper No. A112. Contribution No. 1992 of the Maryland Agricultural Experiment Station (Department of Poultry Husbandry). The author is indebted to Wilson and Company, Inc., Chicago, for 2X gelatin and liver fraction L and to Merck and Company, Inc., Rahway, New Jersey, for the crystalline vitamins.

<sup>1</sup> Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A., *Science*, **101**, 489 (1945).

<sup>2</sup> Briggs, G. M., Jr., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *Proc. Soc. Exp. Biol. and Med.*, **51**, 59 (1942). Briggs, G. M., Jr., Luckey, T. D., Teply, L. J., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **148**, 517 (1943).

<sup>3</sup> Briggs, G. M., *J. Nutr.*, in press.

The inhibition reported in this study cannot be explained solely by the glycine content of the gelatin<sup>4</sup> (Groups 1 and 5). Likewise, the depressing effect of gelatin cannot be due to too high a level of nitrogen in the diet,

Group No.	Supplement to basal diet	Total No. of chicks	Average weight at 4 wks.	No. with black-tongue
			gm.	
1	10% gelatin	30	144	24
2	10% " + 5 mg. nicotinic acid per 100 gm.	23	314	0
3	10% " + 0.1% <i>dl</i> -tryptophane	6	249	0
4	10% " + 0.2% "	24	325	0
5	0.5% <i>l</i> (+)-arginine HCl + 3% glycine	12	274	0
6	As Group 5 + 5 mg. nicotinic acid per 100 gm.	6	296	0
7	" " 5 + 0.2% <i>dl</i> -tryptophane	6	315	0

as shown by the substitution of casein for gelatin in similar rations, which resulted in normal weight gains. Other factors are apparently involved.

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<sup>4</sup> Hier, S. W., Graham, C. E., and Klein, D., *Proc. Soc. Exp. Biol. and Med.*, 56, 187 (1944).

# MODIFICATIONS IN THE ACETOBACTER SUBOXYDANS ASSAY FOR *p*-AMINOBENZOIC ACID\*

Sirs:

A previous communication<sup>1</sup> has discussed the use of *Acetobacter suboxydans* for the assay of the lactone moiety of pantothenic acid. The growth medium for the organism therein described was changed in several respects from those used earlier for *p*-aminobenzoic acid assay,<sup>2,3</sup> in particular to include glucose, norit-treated peptone and liver, and additional norit-treated hydrolyzed casein. These changes produced a greater response when the lactone was added to the medium.

It was thought that this enhanced response might also be exhibited when *p*-aminobenzoic acid was the limiting factor for growth. The results in the accompanying table indicate that this is the case. The  $\beta$ -alanine and *p*-aminobenzoic acid in the medium for lactone assay<sup>1</sup> have been replaced

*Response of Acetobacter suboxydans to p-Aminobenzoic Acid*

PABA per 10 ml.	Galvanometer reading (optical density)	
	Present modified medium	Landy-Streightoff medium
$\gamma$		
0	0.075	0.065
0.002	0.285	0.195
0.004	0.390	0.275
0.006	0.440	0.320
0.008	0.470	0.340
0.010	0.490	0.360

by 200  $\gamma$  of calcium pantothenate per liter. The growth resulting from added *p*-aminobenzoic acid is compared to that obtained on the Landy-Streightoff medium.<sup>3</sup>

With the new medium, various samples of yeast have been assayed for their *p*-aminobenzoic acid content. The values obtained agreed well when calculated at progressively higher assay levels. Recoveries of the added vitamin usually were within  $\pm 15$  per cent of the theoretical values.

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<sup>1</sup> Sarett, H. P., and Cheldelin, V. H., *J. Biol. Chem.*, **159**, 311 (1945).

<sup>2</sup> Landy, M., and Dicken, D. M., *J. Biol. Chem.*, **146**, 109 (1942).

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